GGDEF-EAL Proteins of *Burkholderia pseudomallei*

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LIST OF ABBREVIATIONS

A.xylinum	*Acetobacter xylinum*
AHL	Acyl-homoserine lactone
BLAST	Basic Local Alignment Search Tool
bp	base pair
B. pseudomallei	*Burkholderia pseudomallei*
C. crescentus	*Caulobacter crescentus*
c-di-GMP	Cyclic diguanylic acid
cfu	colony forming unit
DNA	deoxyribose nucleic acid
DGC	diguanylate cyclase
et. al.	*et alter* (and others)
E. coli	*Escherichia coli*
Fig.	figure
GMP	guanosine monophosphate
GTP	guanosine triphosphate
His	Histidine
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani
moi	multiplicity of infection
ORF	open reading frame
O.D.	optical density
PDE	phosphodiesterase

pGpG	phosphoguanylyl- (3'-5')- guanosine
P. aeruginosa	*Pseudomonas aeruginosa*
P. putida	*Pseudomonas putida*
PCR	polymerase chain reaction
QS	quorum sensing
RP-HPLC	reverse phase high performance liquid chromatography
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
S. typimurium	*Salmonella typimurium*
TEM	transmission electron microscopy
V. cholerae	*Vibrio cholerae*
WT	wild type
X. campestris	*Xanthomonas campestris*

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Summary

The Gram-negative bacillus *Burkholderia pseudomallei*, is the causative agent of melioidosis. Its potential threat as a bioterrorism weapon, compounded by the high morbidity and mortality rates of melioidosis, has necessitated the continual research on the pathogenesis of this bacterium.

Recently, a novel guanosine signaling nucleotide, cyclic diguanylic acid (c-di-GMP), has gathered much scientific interest. This ubiquitous second messenger is found in almost all sequenced branches of the phylogenetic tree of *bacteria* and shown to be involved in the regulation of adaptive bacterial functions such as motility, biofilm formation and virulence. In bacteria, the diguanylate cyclase and phosphodiesterase activities of proteins containing the highly conserved GGDEF and EAL domains regulate the intracellular levels of c-di-GMP.

The *B. pseudomallei* genome encodes 16 putative proteins containing the GGDEF-EAL domains. This study focused on two such proteins, CdpA and BPSS0805, which were expressed in both mid-log phase and stationary phase. A comparison of the intracellular c-di-GMP in the gene knockout mutants with that of wild-type *B. pseudomallei* KHW revealed an 8-fold higher c-di-GMP level in the *cdpA* knockout mutant (KHW*cdpA*::Tet). The levels of intra cellular c-di-GMP was restored in *cdpA* complemented mutant, KHW*cdpA*::Tet/pUCP28T-*cdpA* and *cdpA* overexpression strain resulted in a 40% decrease in intracellular c-di-GMP levels. Taken together, these results suggested that CdpA most likely functions as a c-di-GMP phosphodiesterase *in vivo*.

Phenotypic characterization of the mutants revealed that *cdpA* controlled the synthesis of flagella, cell length and swimming motility. Mutation in *cdpA* also increased

cellulose synthesis, cell aggregation and enhanced biofilm formation. The *cdpA* mutant was also significantly attenuated cell invasion and cytotoxicity, thus providing preliminary evidence that high intracellular level of c-di-GMP could inhibit *B. pseudomallei* virulence.

In contrast, *BPSS0805* mutant (KHW*BPSS0805*::Tet) did not significantly alter the level of intracellular c-di-GMP. This mutant also yielded little observable differences in swimming motility, bacteria morphology, cell aggregation, cellulose production and cell invasion and cytotoxicity assays when compared to the wild-type *B. pseudomallei*, thus suggesting functional redundancy of BPSS0805 *in vivo*.

1.0 Introduction

1.1 Melioidosis

Burkholderia pseudomallei is an oxidase-negative, gram-negative environmental saprophytic bacillus found predominantly in moist soils and rice paddies. It was first described by Whitmore and Krishnasawami in 1912 under the name *Bacillus pseudomallei* following its isolation in Rangoon, Myanmar and was later identified as the causative agent of Whitmore's disease (Melioidosis) (White, 2003).

Although mostly prevalent in south-east Asia and Northern Australia, there is mounting evidence that melioidosis is an emerging global problem. In recent years, cases of melioidosis have been documented in non-endemic countries including India, China, Taiwan, Laos and, very recently, Brazil (Peacock, 2006). In Singapore, melioidosis is a disease of growing concern with a record of 84 cases reported between January to September 2004 and a mortality rate of 32.1% (Orellana, 2004). Infection is believed to be primarily acquired via cutaneous or respiratory routes through contact with contaminated soils or water. Clinical manifestations of melioidosis vary greatly and may result in acute pulmonary infection, localized skin infection or even septicemia. This disease is further characterized by formation of abscesses, especially in the lungs and has a latency period ranging from 2 days to 62 years. Several predisposing risk factors such as diabetes, alcoholism and chronic renal diseases are also commonly documented in patients with severe melioidosis (Cheng and Currie, 2005; Wiersinga *et. al.*, 2006).

1.2 *Burkholderia pseudomallei,* the causative agent of melioidosis

The genome sequences of *B. pseudomallei* K96243 and several other *B. pseudomallei* strains were completed recently. The *B. pseudomallei* K96243 genome comprises two chromosomes of 4.07 megabase pairs and 3.17 megabase pairs respectively. Significant functional partitioning of genes between the chromosomes were observed, with the larger chromosome encoding many of the core functions associated with central metabolism and cell growth while the smaller chromosome carries more accessory functions associated with adaptation and survival in different niches (Holden *et al.* 2004; Wiersinga *et. al.*, 2006).

Phenotypically, *B. pseudomallei* is a small (0.8 x 1.5 μm), motile, non-spore forming bacterium (White, 2003). It has a polar tuft of one or more flagella, which is important for its swimming motility and a necessary virulence determinant of *B. pseudomallei* during intranasal and intraperitoneal infection of mice (Chua *et al*., 2003). In the wild, the common location for *B. pseudomallei* in soil is at the root zone of plants whereby the bacteria readily form biofilm at the solid-liquid interface. Furthermore, to survive hostile environmental conditions for prolonged period, *B. pseudomallei* is capable of internalization within amoebic cysts or in the cytoplasm of arbuscular mycorrhizal fungi (Inglis and Sagripanti, 2006).

A facultative intracellular bacterial pathogen that invades a variety of cell types such as macrophages, *B. pseudomallei* is able to sequester within the host cells in a dormant or quiescent state and thus, is intrinsically resistant to multiple classes of antibiotics such as β-lactams, aminoglycosides, macrolides and polymyxins (White, 2003). Currently, ceftazidime-containing regimen remains as the treatment of choice for acute melioidosis but the emergence of fully virulent chloramphenicol- and ceftazidime-resistant strains is a growing cause for concern. In fact, despite treatment

with high-dose ceftazidime, severe melioidosis has a mortality rate of 40%. Besides, *B. pseudomallei* is also capable of acquiring adaptive resistance during a therapeutic course, leading to relapses with similar morbidity and mortality rates to that seen in primary cases (Peacock, 2006). Moreover, due to the relative ease of its weaponization and moderate morbidity and mortality rates, *B. pseudomallei* is now listed as a category B bioterrorism agent by the Centers for Disease Control and Prevention (CDC) (Wiersinga *et. al.*, 2006). This potential threat as a bioterrorism weapon, compounded by the high morbidity and mortality rates of melioidosis, has necessitated the continual research on the pathogenesis of this bacterium.

The pathogenicity of *B. pseudomallei* is dependent on a number of virulence factors including phospholipase C, siderophores and proteins such as haemolysin, lipases and proteases (Yang *et. al*., 1993; Ashdown and Koehler, 1990). Many of these secreted proteins have been found to have cytotoxic and proteolytic activities. MprA protease, for instance, was shown to be involved in the digestion of a variety of eukaryotic proteins substrates and essential for full virulence in a rat model of lung infection (Sexton *et. al.,* 1994). Production of these virulence determinants was strictly regulated and most probably trigger by external environment cues and/or extracellular signals. It is only recently that studies on quorum sensing (QS), a cell-density-dependent mechanism by which bacteria communicate using extracellular signals called autoinducers, has began to shed light on this complex network of regulatory circuits. In *B. pseudomallei,* three LuxI and five LuxR homologues were identified as the main regulators of its quorum sensing system. Mutagenesis of different components of the QS circuitry reduced organ colonization and increased the time to death of aerosolized BALB/c mice (Ulrich *et. al.,* 2004). Our laboratory has also identified the BpsIR quorum sensing system of *B. pseudomallei* and its relevance

to virulence and biofilm formation (Song *et. al.,* 2005). Furthermore, one of the LuxI-LuxR homologues, PmlI-PmlR, was shown to direct the synthesis of *N*-decanoylhomoserine-lactone and regulation of MprA protease production (Valade *et. al.,* 2004).

Interestingly, a few very recent studies demonstrated the close integration between the quorum sensing system and other signaling network such as c-di-GMP signaling in bacteria (Waters *et. al.,* 2008; Williamson *et. al.,* 2008; Zhou *et. al.,* 2008). Thus, given the importance of regulatory network in pathogenesis, further study in this field could one day lead to improvements in the control and prevention of diseases such as melioidosis.

1.3 Cyclic di-GMP signaling in bacteria

The bacterial signal transduction network is a complex array of numerous interacting components that collectively facilitate the conversion of specific environmental cues to appropriate bacterial physiological responses. These responses which are often receptor mediated, utilize diffusible small molecules including cyclic nucleotides such as adenosine 3',5'-cyclic monophosphate (cAMP), guanosine 3', 5'-cyclic monophosphate (cGMP) and guanosine-3,5-bis(pyrophosphate) (ppGpp) as second messengers. Since early 1970s, several of these molecules have been extensively studied and were shown to play diverse roles in the regulation of basic bacteria physiology. cAMP, for instance, allosterically activates a transcription factor, catabolite regulation protein (CRP) to regulate catabolic operons as alternative carbon sources and other cellular processes (Pastan and Pernman, 1970). Another second messenger cGMP is known to be involved in the regulation of bacterial swimming behavior during chemotaxis (Black *et. al.,* 1980). In addition, ppGpp was shown to

play a key role in bacterial stringent response under starvation conditions (Cashel, 1975).

In the recent years, another novel guanosine signaling nucleotide, cyclic diguanylic acid (c-di-GMP), has gathered much scientific interest. This low molecular-weight, heat stable guanyl oligonucleotide composing of two GMP residues was first discovered in the allosteric activation of a cellulose synthases complex in *Gluconacetobacter xylinus* (Ross *et al.,* 1987) and later implicated in many bacterial phenotypes including regulation of bacterial motility, exoploysaccharide production, biofilm development, regulation of virulence factors production and other phenotypes (Fig. 1).

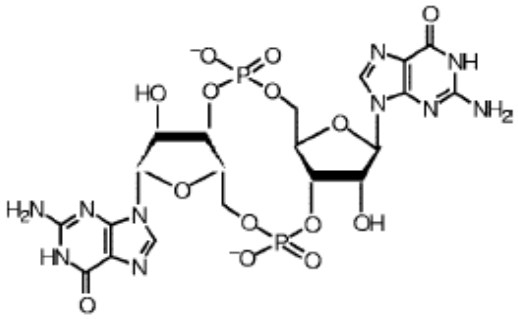


Fig. 1 C-di-GMP consists of two cGMP molecules joined by a 3', 5'-phosphodiester bond.
This ubiquitous bacterial intracellular signaling molecule has attracted great scientific interest and is conceived to play an important role in several phenotypes such as bacterial motility, exopolysaccharide production, biofilm formation, virulence factors production. (Adapted from Fouhy *et. al.,* 2006).

In bacteria, c-di-GMP is synthesized from GTP by a class of enzymes containing GG(D/E)EF domains known as diguanylate cyclases (DGCs) and hydrolyzed by phosphodiesterases (PDEs) proteins containing the EAL / HD-GYP domains to 5'-phosphoguanylyl- (3'-5')- guanosine (pGpG) and subsequently broken down into guanosine monophosphate (GMP). The regulatory actions of c-di-GMP can be targeted at the transcriptional, translational or posttranslational levels, acting either

allosterically with specific enzymes such as cellulose synthases (Ross *et al.,* 1987), through another effector proteins such as PilZ domain protein (Ryjenkov *et. al.,* 2006), or its homolog DgrA (Christen *et. al.,* 2007), regulation of transcription factor such as FleQ in *Pseudomonas aeruginosa* (Hickman and Harwood, 2008), or possibly interact directly base pairing with other nucleic acids such as mRNA or small regulatory RNA molecule as exemplified by *Escherichia coli* GGDEF-EAL protein, CsrD that controls the degradation of global regulatory RNAs (Suzuki *et. al.,* 2006). In addition, a recent class of mRNA domains known as riboswitches was shown to sense changes in c-di-GMP and regulates the expression of downstream genes associated with phenotypes such as motility, biofilm formation and bacteria virulence (Sudarsan *et. al.,* 2008) (Fig. 2).

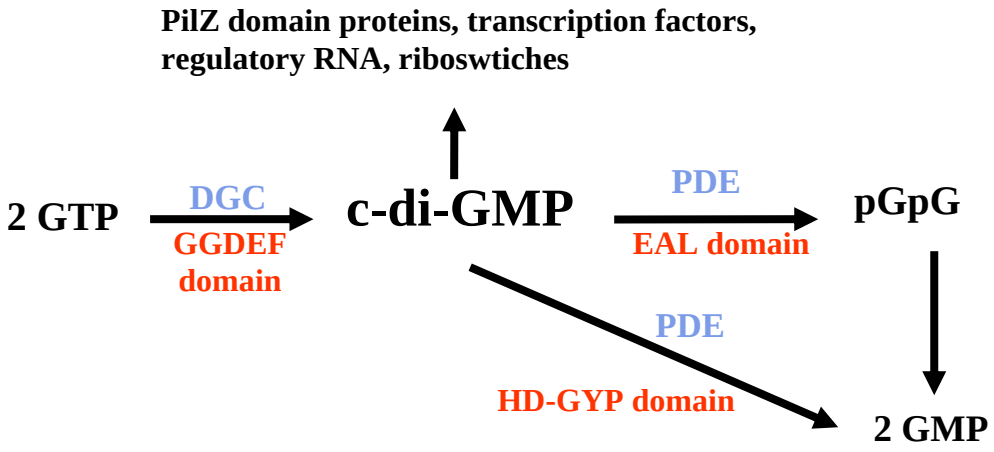


Fig. 2. Cyclic-di-GMP regulatory pathway
Cyclic-di-GMP is synthesized from GTP by diguanylate cyclases containing GG(D/E)EF domains (Pfam Accession No. PF00990) and hydrolyzed by phosphodiesterases containing the EAL / HD-GYP domains (Pfam Accession No. PF00563) to pGpG and subsequently broken down into GMP. C-di-GMP can bind to its receptor proteins such as PilZ domain protein (Pfam Accession No. PF07238), which could act as molecular switches through the interaction with other protein partners or allosterically interacting with effector enzymes.

1.4 Bacterial Motility

It is now well established that bacterial motility is seldom a random event (Szurmant and Ordal, 2004). The bacterium depends on several forms of movement for its translocation ranging from swimming, swarming, gliding, twitching or floating (Jarrell and McBride, 2008). Advantages of locomotion to bacterium are numerous, including the ability to move towards favorable conditions and avoid detrimental ones, colonization of new niches, and interact or compete against other microorganisms (Fenchel, 2002). In fact, bacteria are able to sense chemical signals in their environment and alter their motility machineries to move towards attractants or away from repellents by a process known as chemotaxis. Chemotaxis involves a complex signal transduction mechanism that includes a membrane-bound chemoreceptor protein and cytoplasmic regulatory components that tightly coupled

the environment cues to flagellar mediated swimming motility (Silversmith and Bourret, 1999).

Swimming motility is perhaps the most intensive studied motility in bacteria and can move the cell at a speed of up to 160µm per second (Lambert *et. al.,* 2006). This movement is commonly associated with the bacterial flagellum, which consists of three main substructures - the basal body, the filament and the hook. The basal body of the flagellum anchors the structure in the cell envelope and contains the motor. The filament, which is approximately 20 nm in diameter, extends many cell lengths from the cell surface and acts as the propeller and the hook, which connects the basal body and the filament and acts as a universal joint (Minamino and Namba, 2004). In *B. pseudomallei*, the swimming activity is largely dependent on the expression of flagella as the *fliC* mutant clearly displayed a non-motile phenotype (Chua *et. al.,* 2003).

Another form of bacterial movement is the swarming motility. This organized surface movement on certain solid media is usually associated with close cell to cell contact and involved numerous flagella. In addition, swarming is often accompanied by a change in cell morphology, with swarmer cells being elongated and more flagellated (Fraser and Hughes, 1999). The swarming motility is observed in several bacteria species including *E. coli, S. typimurium* and *V. chlorae* though studies in our lab did not observe swarming motility in *B. pseudomallei* (Song, 2003)

Besides flagellar mediated movement, bacteria such as *Neisseria gonorrhoeae,* which lack rotary flagella, can move through non flagellar mediated mechanisms such as type IV pili (Tfp) mediated twitching motility. This bacterial movement is often crucial for host colonization and other forms of complex bacterial behavior including biofilm formation. The mechanism of twitch involves the Tfp, which extend from the

cell poles and through its extension, attachment to a surface and retraction propel the cell forward (Merz *et al.,* 2000). In addition, bacteria can move across surfaces through non flagellar mediated gliding movement. To date, the exact mechanism involved in this motility is relatively unknown, though studies in *Flavobacterium johnsoniae* has shown cell surface proteins such as GldA, FtsX and SprB are crucial for its gliding (Nelson *et al.,* 2008).

1.4.1 C-di-GMP is a key regulator of transition from motility to sessility

The first direct evidence of c-di-GMP regulation of bacterial motility was shown by Huang and his team in 2003. A mutation in the *P. aeruginosa* GGDEF-EAL protein, *fimX* led to increased intracellular c-di-GMP levels and an inhibition of the bacteria's twitching motility (Huang *et al.,* 2003). C-di-GMP was also shown to inversely regulate flagellar motility in pathogenic bacteria such as *Salmonella typhimurium* and *Vibrio cholerae*. Simm *et al* demonstrated that overexpression of PDEs resulted in decreased c-di-GMP levels and enhanced bacterial motility (Simm *et al.,* 2004). Subsequently, it was also shown in *V. cholerae* that overexpression of DGCs led to increased c-di-GMP levels and significantly inhibited both swarming and swimming motility (Beyhan *et. al.,* 2006).

Interestingly, researchers have shown that c-di-GMP can act on distinct levels of the control hierarchy to regulate bacterial motility. In different bacteria, transcriptional expression of motility genes, posttranslational mechanisms, including organelles assembly, and inhibition of motor functions were regulated by intracellular c-di-GMP levels (Wolfe and Visick, 2008). In whole-genome transcriptome analysis of *V. chlorea*, an increase in levels of c-di-GMP was shown to decrease motility through transcriptional downregulation of flagellar genes expression (Beyhan *et. al.,*

2006). In *V. parahaemolyticus,* two loci encoding GGDEF-EAL proteins, *scr ABC* operon and *scr G* (*s*warming and *c*apsular polysaccharide gene *r*egulation) were shown to regulate lateral flagellar gene expression, capsular polysaccharide (CPS) production and swarming phenotype. When grown on a surface, *V. parahaemolyticus* differentiates from a swimmer cell that is polarly flagellated to a swarmer cell that is laterally flagellated for efficient motility. Mutations in *scrABC* operon and *scrG* resulted in increased c-di-GMP levels and led to enhanced capsular polysaccharide production and severe swarming. Two-dimensional thin layer chromatography (TLC) analysis of nucleotide pools of *scrC* and *scrG* overexpression strains and their respective null mutants identified both proteins as a c-di-GMP phosphodiesterase (Boles and McCarter; Kim and McCarter, 2007; Ferreira *et. al.,* 2008).

Recent studies have also revealed the roles of c-di-GMP in posttranslational regulation of motility. For instance, in V. *fischeri*, the overexpression of DGC, MifA did not alter the levels of flagellin transcript although motility and flagellin protein level is significantly affected (O'Shea *et. al*., 2006). In another study, a GGDEF-EAL protein, MorA in *Pseudomonas* species was shown to alter c-di-GMP levels and consequently control the timing of flagellar development in *P. putida* and affect bacterial motility (Choy *et. al.,* 2004). Intracellular c-di-GMP was increased in an *E. coli* null mutant of the EAL domain protein, *yhjH*, resulting in significant inhibition of swimming motility. This motility defect was restored by a secondary mutation in *ycgR*, which encoded a c-di-GMP receptor PilZ domain and a regulator of flagellum-based motility in a c-di-GMP dependent manner (Ryjenkov *et. al.,* 2006). In addition to regulation of flagella based motility, c-di-GMP can also affect motility through other cell surface apparatus such as Tfp. For instance, in *P. aeruginosa,* the Tfp's

motor functions were shown to be governed by FimX, a PDE protein that regulate the levels of intracellular c-di-GMP (Kazmierczak *et al.,* 2006).

1.5 Biofilm formation in bacteria

In the natural environment, most, if not all, bacteria generally exhibit two distinct modes of behavior, either as free swimming planktonic cells or sessile surface attached communities surrounded by an extracellular polysaccharide (EPS) matrix. This sessile community of single or multiple populations of bacteria characterized by cells that are irreversibly attached to a substratum or interface or to each other and embedded in a matrix of extracellular polymeric substances and exhibit an altered phenotype with respect to growth rate and gene transcription is generally defined as biofilm (O'Toole *et al*., 2000).

As proposed by Stoodley *et. al.*, (2002), the formation of biofilm generally involves five main stages. In the first stage, free swimming planktonic cells move together and begin their initial attachment to the surface. Subsequently, the bacteria form microcolonies and initiate the production of EPS, which resulted in a more firmly "irreversible" attachment. Next, as cells are firmly attached to the surface, the early development and maturation of biofilm architecture will commence. And the last stage usually involves the dispersion of single cells from the biofilm (Fig. 3)

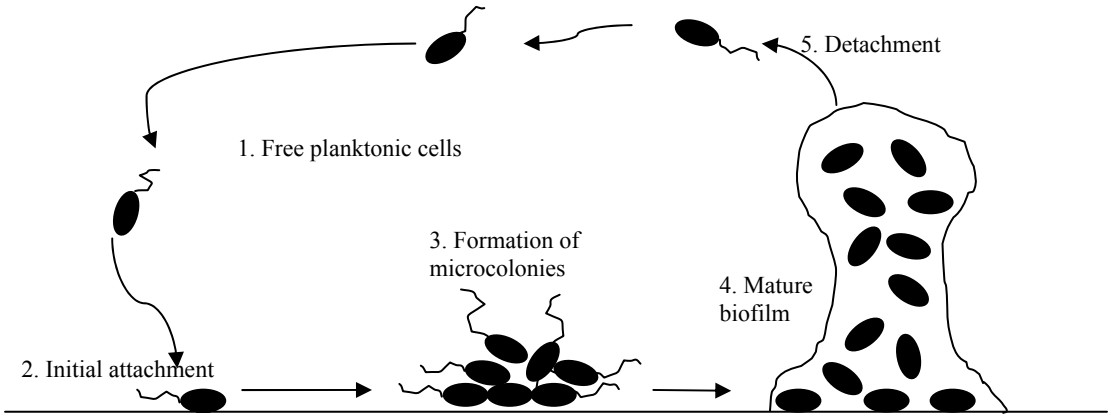


Fig. 3. The 5 stages biofilm development model.
Stage 1: Bacterial swims freely in medium using flagellar powered motility. Stage 2: Initial attachment to surface Stage 3: Formation of microcolonies and production of EPS. Stage 4: Maturation of biofilm architecture Stage 5: Detachment of single cells from biofilm. Adapted with permission from Stoodley *et. al.*,(2002).

By forming biofilm, bacteria can increase their resistance to antimicrobial agents by several hundred folds higher than their free-living counterpart and in general, their survivability in hostile environments (Gilbert *et. al.,* 1997). Biofilm formation is hence, a crucial step in the pathogenesis of many sub-acute and chronic bacterial infections such as infectious kidney stones, bacterial endocarditis, and cystitis fibrosis airway infections (Parsek and Singh, 2003; Hall-Stoodley, 2004; del Pozo and Patel, 2007).

Similarly, in *B. pseudomallei,* biofilm formation is critical for its survivability in harsh environmental conditions (Vorachit *et al.,* 1993). Bacteria within the biofilm slow down their growth rates, thus increasing their inherent resistance to the actions of disinfectants and antibiotics (Inglis and Sagripanti, 2006). In addition, in the initiation of biofilm formation, *B. pseudomallei* was shown to produce exopolysaccharide materials that constitute a highly hydrated glycocalyx that facilitate the formation of microcolonies, thus allowing them to adhere readily and non-specifically to abiotic surfaces (Vorachit *et. al.*, 1995). The subsequent

maturation of *B. pseudomallei* biofilm is cell density dependent and positively regulated by its BpsIR quorum sensing systems (Song *et. al.,* 2005).

The formation of biofilm is controlled by multiple convergent signaling pathways and is highly influenced by external environment cues, including temperature, osmolarity, pH, iron, and oxygen (Stoodley *et. al.*, 2002). For example, *V. chlorea,* switches from a sessile biofilm community on the host plankton chitin exoskeleton to a free swimming form upon entering its mammalian host (Reguera and Kolter, 2005). In another example, *X. campestris* exists in the planktonic form during vascular invasion but switches to form biofilm during the colonization of leaf surfaces (Crossman and Dow, 2004).

1.5.1 C-di-GMP is a key regulator of biofilm formation

A role for c-di-GMP in the regulation of bacterial biofilm formation was first proposed by two separate groups through the characterization of GGDEF – EAL domain proteins in *V. parahaemolyticus* and *P. aeruginosa* (Boles and McCarter, 2002; D'Argenio *et. al*., 2002). D'Argenio and his team working on insertional mutants of *P. aeruginosa* found a link between the response regulator GGDEF protein, WspR and autoaggregation. The aggregation phenomenon was linked to aberrant regulation of *cup* genes that encoded a putative fimbrial adhesin which was also required in wild-type cells for biofilm formation (D'Argenio *et al.*, 2002). Subsequently, a comprehensive analysis of *P. aeruginosa* genes encoding the enzymes of c-di-GMP metabolism revealed that a *P. aeruginosa* transposon mutant with a non-functional WspR exhibited decreased formation of biofilm whereas overexpression of *wspR* increased biofilm formation (Hickman *et. al.,* 2005; Kulesekara *et al.*, 2006). In *P. fluorecens,* the WspR is required for the

overproduction of attachment factors and likewise, a mutation in WspR abolished cellular attachment and exopolysaccharide production (Malone *et. al.,* 2007).

The regulation of biofilm formation by c-di-GMP was also verified by studies in a number of different bacteria including *V. cholerae (*Tischler and Camilli, 2004; Lim *et. al.,* 2006)*, S. typhimurium* (García *et. al.*, 2004)*, Shewanella oneidensis* (Thormann *et. al*., 2006)*, P. putida* (Gjermansen *et al.,* 2006). Increased levels of c-diGMP were associated with enhanced biofilm formation while decreased intracellular levels of c-diGMP resulted in defective biofim initiation. High concentrations of intracellular c-di-GMP commonly led to decreased motility, increased expression of adhesive matrix components and cell aggregation which are fundamental components essential for biofilm development (Simm *et al.,* 2004; Cotter and Stibitz, 2007).

Specifically, the association between c-di-GMP and biofilm formation is well studied in *V. cholerae.* C-di-GMP was found to regulate *Vibrio* polysacharide (VPS) production, a requirement by *V. cholerae* for biofilm formation. VieA, a c-di-GMP PDE, represses the transcription of VPS genes involved in biofilm formation through controlling the intracellular levels of c-di-GMP (Tischler and Camilli, 2004). Lim *et al* subsequently identified mutations in genes encoding three other GGDEF-EAL proteins, CdgC, RocS and MbaA, when compared to their wild types counterparts, resulted in increased biofilm forming capacity and biofilms with different architectures. In addition, as phenotypes of these mutants, though similar, are actually distinguishable, the authors constructed double knockout mutants to further demonstrate that c-di-GMP regulation of biofilm in *V. cholerae* is a complex and interlinked network (Lim *et. al.,* 2006). However, it was interesting to note that in *Staphylococcus aureus,* extracellular c-di-GMP actually inhibits, instead of promote,

intercellular adhesive interactions and biofilm formation (Karaolis *et. al.,* 2005). Taken together, though the exact mechanism by which c-di-GMP inhibit biofilm is still unclear, it is obvious that given the significant effects of c-di-GMP on biofilm formation in a wide range of bacteria species, this second messenger could potentially be a antimicrobial drug lead.

1.6 C-di-GMP is a key regulator of virulence in bacteria

Besides the regulation of virulence associated phenotypes such as motility, c-di-GMP can also directly modulate the pathogenic capacity of bacterial pathogens and production of virulence factors. As the production of virulence factors by pathogenic bacteria is tightly regulated and occurs in response to environmental signals, several recent studies have implicated the roles of c-di-GMP in bacterial virulence studies.

For instance, the mutation of *V. cholera* VieA repressed the major virulence gene transcriptional activator, *toxT* and *ctxA,* which encodes cholera toxin and therefore the attenuation of virulence in mice (Tischler and Camilli, 2005). Besides VieA, a separate study has shown that another c-di-GMP phosphodiesterase, CdgC also positively regulates various virulence genes including *ctxAB* expression in *V. cholera* (Lim *et al.,* 2007). Similarly, a systematic analysis of *P. aeruginosa* GGDEF-EAL domain mutants revealed that high levels of c-di-GMP led to decrease in virulence associated traits and attenuated virulence in a mouse infection model **(**Kulasakara *et. al.,* 2006). Also, Hisert and his colleagues showed that in *S. typimurium,* a mutation in CdgR, an EAL domain protein, was shown to decrease bacterial resistance to hydrogen peroxide and increase its vulnerability to killing by macrophages (Hisert *et al.,* 2005). In addition, a proteomic strategy was used to study the influence of c-di-GMP phosphodiesterase, PigX, on the virulence factors

production in *Serratia* strain ATCC 39006. In this study, the authors showed that mutation of PigX resulted in increased levels of OpgG, which regulate the production of plant cell wall degrading enzymes and consequently produced a hypervirulent phenotype (Fineran *et. al.,* 2007).

Overall, these studies presented evidence that the regulation of virulence by c-di-GMP is more often than not, a complex and multifaceted process. Not only does the c-di-GMP control phenotypes linked to virulence, this second messenger can directly regulate virulence in a number of pathogenic bacteria by inhibition of virulence gene expression.

1.7 GGDEF – EAL proteins in bacteria

Given the key roles of c-di-GMP in regulating many bacterial phenotypes including regulation of bacterial motility, biofilm development and regulation of virulence factors production, there is a compelling need to further investigate the involvement of GGDEF-EAL proteins in turnover of this second messenger (Tamayo *et. al.,* 2007)..

The turnover of c-di-GMP was first described by Tal and his group through the isolation of genes that controlled c-di-GMP levels in *G. xylinus*. Three operons involved in c-di-GMP metabolism were identified and found to encode proteins that contain conserved motifs Gly-Gly-Asp-Glu-Phe (GGDEF) and Glu-Ala-Leu (EAL). These GGDEF and EAL domains were arranged in tandem, with the approximately 250 amino acid EAL domain located at C terminal of the approximately 170 amino acid GGDEF domain (Tal *et. al.,* 1998).

In a study conducted by Simm *et al*, the expression of a GGDEF domain protein, AdrA, was shown to be directly correlated to the level of intracellular c-di-

GMP in *S. Typhimurium*. On the contrary, the overexpression of YhjH, a EAL domain protein, led to a downregulation decrease in its level of intracellular c-di-GMP concentrations (Simm *et. al.,* 2004).

The involvement of GGDEF and EAL proteins in c-di-GMP production and degradation respectively was also clearly established by the *in vitro* analysis of several GGDEF and EAL domains. Six randomly chosen GGDEF proteins of different branches of the bacterial phylogenetic tree were overexpressed by Ryjenkov and his team *in vitro* and were shown to possess DGC activity (Ryjenkov *et. al.,* 2005). In a separate study, purified full length *E. coli* YahA protein and its EAL domain were overexpressed and shown *in vitro* to specifically degrade c-di-GMP in the presence of Mg^{2+} and Mn^{2+} (Schmidt *et. al.,* 2005). At around the same period, Tamayo and his colleagues also undertook a study to demonstrate that the EAL domain of *V. cholera* protein, VieA is responsible for its c-di-GMP phosphodiesterase activity *in vitro* (Tamayo *et. al.,* 2005).

However, the EAL domain was not the sole c-di-GMP phosphodiesterase in bacteria. In 2003, a second unrelated protein domain, HD-GYP was identified in *Xanthomonas campestris* as a c-di-GMP phosphodiesterase (Dow *et. al.,* 2003). HD-GYP belongs to the subgroup of metal dependent phophohydrolase superfamily and unlike EAL protein, HD-GYP proteins directly hydrolyses c-di-GMP into guanosine monophosphate (GMP) (Ryan *et. al.,* 2006).

A closer examination of the architecture of these proteins reveals that although GGDEF domain alone is sufficient to encode DGC activity, its activities are often regulated by adjacent sensory protein domains (Ryjenkov *et al.,* 2005). The majority of these proteins were linked to signal sensor domains, which allowed them to detect

environmental signals such as oxygen, light, small ligands and membrane-derived signals (Galperin *et. al.*, 2001; Galperin, 2004).

In some bacteria, instead of adjacent sensory domains, the GGDEF or EAL domains are involved in these regulatory roles. For instance, the catalytically inactive GGDEF domain of *C. crescentus* CC3396 activated its N terminal EAL domain by binding to GTP (Christen *et. al.,* 2005). In another *C. crescentus* protein PleD, its GGDEF domain folding closely resembles that of class III nucleotidyl cyclases such as bacterial and eukaryotic adenylyl cyclases, suggesting that phosphorylation mediated dimerization of the DGC domains are crucial for its activation. It also proposed an inhibition model whereby c-di-GMP exhibit product feedback inhibition of the allosteric-binding site on the DGC domain of PleD (Chan *et. al.,* 2004; Christen *et al*., 2006; Paul *et. al.*, 2007).

Characterization of the GGDEF – EAL proteins provided the basis for the rapidly accumulating interest in this field. Moreover, current advances in microbial genomic sequencing revealed that these proteins are found in almost all sequenced branches of the phylogenetic tree of *Bacteria,* though notably absent in genomes of any *Archaea* or *Eukarya*. Interestingly, the number of GGDEF-EAL domain proteins encoded in bacteria genome is highly variable, ranging from none in *Heliobacter pylori,* 40 in *P. aeruginosa* to almost 100 of them in *V. vulnificus* (Galperin *et. al.*, 2001; Galperin, 2005). This diverse variability in the number of GGDEF – EAL proteins encoded in different bacteria raised the questions of how the bacteria is able to coordinate the expression and activity of these proteins to tightly control c-di-GMP and more importantly, how these differences affect the phenotypes regulated by this ubiquitous secondary messenger.

1.8 Objectives of the project

The intrinsic drug resistance of *B. pseudomallei*, compounded by its potential threat as a bioterrorism weapon, has necessitated further research to gain a better understanding of the physiology of this bacterium. Although melioidosis is an important infectious disease in this region, our understanding of molecular pathways, especially its signaling cascade is still limited. To date, despite numerous studies on c-di-GMP in many Gram negative pathogens, there is still no published work exploring this complex signaling pathway in *B. pseudomallei.*

The aims of this project are two-fold: (1) to identify the putative genes encoding *B. pseudomallei* GGDEF-EAL proteins and understand their roles in the turnover of c-di-GMP, and (2) to characterize the involvement of the GGDEF-EAL encoding genes in several common phenotypes regulated by c-di-GMP including bacterial motility, flagella synthesis, autoaggregation, cellulose production, biofilm formation and virulence. Overall, we would like the study to further the understanding of the complex nature of c-di-GMP signaling and its roles in the pathogenesis of *B. pseudomallei.*

2.0 Materials and methods

2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. pseudomallei* isolate KHW was from the collection of E. H. Yap at the Department of Microbiology, National University of Singapore. Unless otherwise stated, *B. pseudomallei* and *E. coli* were cultured at 37°C in Luria–Bertani (LB) broth with shaking at 100 rpm or on LB agar medium (Becton Dickinson, Cockeysville, Md.).

Where appropriate, the antibiotics concentrations used for *E. coli* were as follows: ampicillin, 100 μg/ml; gentamicin, 30 μg/ml; trimethoprim, 25 μg/ml; kanamycin, 25μg/ml; chloroamphenicol, 34 μg/ml; tetracycline, 10 μg/ml. Antibiotics for used *B. pseudomallei* were at the following concentrations: kanamycin, 200 μg/ml; trimethoprim, 100 μg/ml; tetracycline, 25 μg/ml. All antibiotics were purchased from Sigma (St. Louis, Mo.).

2.2 Cell lines

Human monocycte-like cell line THP-1 (ATCC, TIB-202) was maintained with RPMI 1640 (Sigma, ST. Louis, MO), supplemented with 10% Fetal Calf Serum (FCS, Hyclone Laboratories, Logan, UT), 2mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (complete RPMI). Human lung carcinoma epithelial cell line A549 (ATCC, CCL-185) was maintained in DMEM (Sigma, ST. Louis, MO) complete media with 10% FCS. The cells were grown at 37°C in the presence of 5% CO_2 and passaged every 3 – 4 days at a ratio of 1:10.

2.3 *In-silico* sequence analysis

The nucleotide sequences of GGDEF-EAL proteins of *B. pseudomallei* strain K96243 were obtained from the Sanger website. (http://www.sanger.ac.uk/Projects/B_pseudomallei). Additional sequences of GGDEF-EAL proteins were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The *in silico* domain architecture analysis of these proteins was carried out using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The prediction of the transmembrane domain was carried out using the TMpred program (http:// www.ch.embnet.org/ software/TMPRED_form.html) (Hofmann and Stoffel, 1993). The bl2seq program, (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) was used to provide pair-wise comparison of translated nucleotide sequences (Altschul *et al*., 1997). Prediction of the putative *B. pseudomallei cdpA* and *BPSS0805* promoters was carried out using the Neural Network Promoter Prediction (NNPP) program for prokaryotes (http://www.fruitfly.org/seq_tools/ promoter.html).

2.4 DNA and RNA manipulations

Routine DNA manipulations (restriction endonuclease digestion, ligation, agarose gel electrophoresis, transformation) were performed according to standard techniques described by Sambrook *et al.* (1989). Restriction enzyme (RE) digestion was performed in a total volume of 10 µl with 10 units of RE and incubated at 37°C for 2 h with its respective buffer. The vectors used and its inserts were cut to produce compatible ends for ligation. Ligation of insert to plasmid vector was carried at 16°C for 18 h using T4 DNA ligase (Promega, USA). *E. coli* DH5α competent cells were transformed with the ligation products via electroporation at 1.8 kV. Plasmid DNA

was isolated using QIAprep spin miniprep kit (Qiagen, USA) according to the manufacturer's protocol.

RNA isolation was carried out using RNeasy Mini kit in combination with RNAprotect Bacteria Reagent (Qiagen, USA). Briefly, the bacteria were grown in AB medium containing 0.2% glucose (w/v) and 0.5% casamino acids with shaking at 100 rpm for 24 h. The stationary-phase bacterial cultures were enzymatically lysed and total RNA from the cell pellets was isolated as per manufacturer's instructions. To remove contaminating DNA, total RNA was incubated with RNase-free DNase 1 recombinant (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR was performed using Access RT-PCR kit (Promega, USA) according to the manufacturer's instructions. The list of RT-PCR primers used is described in Table 2.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Reference or source
***B. pseudomallei* strains**		
KHW	Virulent clinical isolate	E.H. Yap, NUS
KHW*cdpA*::Tet	Derivative of KHW with *cdpA* gene disrupted by the insertion of a 1743 bp tetracycline cassette from pFTC1; Tet^r, Gen^r	This study
KHW*cdpA*::Tet/pUCP28T-*cdpA*	KHW*cdpA*::Tet complemented in *trans* with pUCP28T*cdpA* plasmid; Tet^r, Tmp^r, Gen^r	This study
KHW/pUCP28T-*cdpA*	KHW carrying pUCPT28T-*cdpA* plasmid; Tmp^r, Gen^r	This study
KHW*BPSS0805*::Km	Derivative of KHW, with *BPSS0805* gene disrupted by the insertion of a 1849bp kanamycin cassette from pUT5; Km^r, Gen^r	This study
KHW*BPSS0805*::Km/pUCP28T-*BPSS0805*	KHW*BPSS0805*::Km complemented in *trans* with pUCP28T-*BPSS0805* plasmid; Km^r, Tmp^r, Gen^r	This study
KHW/pUCP28T-*BPSS0805*	KHW carrying pUCPT28T-*BPSS0805* plasmid; Tmp^r, Gen^r	This study
KHW*cdpA*::Tet/pUCP28T-*BPSS0805*	KHW*cdpA*::Tet strain complemented in *trans* with pUCP28T-*BPSS0805*. Tet^r, Tmp^r, Gen^r	This study
***E. coli* strains**		
DH5αλpir	DH5α with a λ prophage carrying the gene encoding the p protein; Kan^S, Tmp^S, Gen^S	Miller and Mekalanos, 1988
HB101/pRK600	Helper strain; containing pRK600 for triparental mating; Cm^r	de Lorenzo and Timmis, 1994

Plasmids		
pFTC1	Tetracycline resistance FRT vector; Tet^r	Choi *et. al*., 2005
pUT5	Source of kanamycin resistance cassette; oriR6K mobRP4; Km^r, Amp^r	de Lorenzo *et. al.,* 1990
pUCP28T	Broad-host-range vector; IncP OriT; pRO1600; *ori* Tmp^r	West *et al.,* 1994
pJQ200mp18	Mobilizable suicide vector for allelic exchange; *traJ, sacB,* Gen^r	Quandt and Hynes, 1993
pJQ200mp18*cdpA*	pJQ200mp18 containing the 1.8 kb BamH1 digested *cdpA* PCR product. Gen^r	This study
pJQ200mp18*cdpA*::Tet	pJQ200mp18 carrying *cdpA*::Tet fragment. The 2.1-kb Tet resistance cassette from pFTC1 was inserted into the AccIII site within *cdpA* coding sequence; Gen^r, Tet^r	This study
pUCP28T-*cdpA*	pUCP28T carrying the full-length *cdpA* and its promoter; Tmp^r	This study
pJQ200mp18*BPSS0805*	pJQ200mp18 containing the 1.8 kb BamH1 digested *BPSS0805* fragment. Gen^r	This study
pJQ200mp18*BPSS0805*::Km	pJQ200mp18 carrying *BPSS0805*::Tet fragment. The 1.8-kb Km cassette from pFTC1 was inserted into the *Bsm*I site within *BPSS0805* coding sequence; Gen^r, Km^r	This study

2.5 Mutagenesis and Complementation

2.5.1 Construction of isogenic KHW*cdpA*::Tet and KHW*BPSS0805*::Km mutants

B. pseudomallei isolate KHW, a virulent clinical isolate previously described in our laboratory (Chua *et. al*., 2003), was used as the parental strain for the construction of isogenic mutants and its complement and overexpression strains. Primers used in this study are described in Table 2.

KHW*cdpA*::Tet mutants were generated by insertion mutagenesis of the *cdpA* gene. Full-length *B. pseudomallei cdpA* gene was first cloned into the suicide vector, pJQ200mp18 (Quandt and Hynes., 1993) using the primer pair: pJQ*cdpA*(BamHI) F - pJQ*cdpA*(BamHI) R. A 2.1-kb tetracycline resistance (Tet^r) cassette from pFTC1 (GenBank accession no. AY712950) (Choi *et. al*., 2005) was then inserted into the *Acc*III restriction site within *cdpA*. Next, the pJQ200mp18*cdpA*::Tet plasmid was transformed into *E. coli* DH5αλpir (N. Judson, Gibco-BRL) and subsequently introduced into *B. pseudomallei* KHW by triparental conjugation using a helper strain *E. coli* HP101/pRK600 as described by de Lorenzo et al (de Lorenzo *et. al*., 1990). After which reciprocal recombinants which had undergone allelic exchange were selected by plating the conjugation mixture on LA plates supplemented with 25 μg/ml of tetracycline, 100 μg/ml streptomycin and 5% sucrose. Exconjugants harbouring the Tet cassette disrupted *cdpA* were identified by PCR using primer pair: *cdpA*::Tet(ver) F and *cdpA*::Tet(ver) R, which yielded a 2.5 kb fragment instead of a 375 bp fragment in the parental *B. pseudomallei* KHW.

The absence of *cdpA* gene expression was confirmed by RT-PCR. In brief, 120 ng of total RNA was isolated from stationary phase KHW and KHW*cdpA*::Tet mutant cultured in AB medium containing 0.2% glucose (w/v) and 0.5% casamino

acids, using RNeasy Mini kit in combination with RNAprotect Bacteria Reagent (Qiagen, USA). RT-PCR was carried out using Access RT-PCR kit (Promega, USA) with the primer pair: *cdpA*(RT-PCR) F and *cdpA*(RT-PCR) R. The PCR conditions were optimized for amplification of fragment size of less than 1kb (PCR extension time of 1 min) to allow for the amplification of a 626 bp *cdpA* PCR product in wild-type KHW but not in KHW*cdpA*::Tet mutant to indicate the successful *cdpA*–null mutation. RT-PCR of 16*SrDNA* using primer pair: 16SrDNA F and 16Sr DNA R was included as an internal control and to check for DNA contamination.

The procedure used for construction of the KHW*BPSS0805*::Km mutant was similar to that of KHW*cdpA*::Tet mutant described above. The *BPSS0805* gene was first disrupted by inserting a 1.8-k.b kanamycin resistance (Km^r) cassette into BsmI site within the coding sequence of *BPSS0805*. Reciprocal recombinants were selected by plating the conjugation mixture on LA supplemented with 200 μg/ml of kanamycin, 100 μg/ml streptomycin and 5% sucrose. Exconjugants harbouring a BPSS0805 gene disrupted with a kanamycin-resistance cassette were detected by PCR using primer pair: *BPSS0805*::Km(ver) F and *BPSS0805*::Km(ver) R as a 2.5 kb product as opposed to a 711 bp product for undisrupted *BPSS0805* gene in the parental *B. pseudomallei* KHW.

The absence of *BPSS0805* gene expression was confirmed by RT-PCR using the primer pair: *BPSS0805* (RT-PCR) F and *BPSS0805* (RT-PCR) R as described above. The PCR conditions employed were optimized for the amplication of PCR fragment of less than 1kb in size to allow for the differentiation of the null mutation. The presence of a 658 bp *BPSS0805* PCR product in wild type *B. pseudomallei* KHW but none in KHW*BPSS0805*::Km mutant confirmed the successful *BPSS0805*–null

mutation. RT-PCR of 16*SrDNA* using primer pair: 16S rDNA F and 16S rDNA R was included as an internal control and to check for DNA contamination.

2.5.2 Construction of *cdpA* and *BPSS0805* complementation and KHW/pUCP28T-*cdpA* and KHW/pUCP28T-*BPSS0805* strains

The full length *B. pseudomallei cdpA* gene and its upstream promoter were amplified from the genomic DNA of KHW using PCR primer pairs: pUCP28T*cdpA* F and pUCP28T*cdpA* R with Expand Long Template PCR system (Roche Diagnostics GmbH, Mannheim, Germany). The 4-k.b. PCR product was ligated into the poly(T) tailed, broad host range vector, pUCP28T and transformed into *E. coli* DH5αλpir. Subsequently, the pUCP28T-*cdpA* plasmid was introduced into *B. pseudomallei* KHW*cdpA*::Tet mutant and wild type KHW via triparental conjugation to generate *cdpA* complement and KHW/pUCP28T-*cdpA* strains respectively. Exconjugants were selected on LA plates supplemented with 100 μg/ml trimethoprim and 100 μg/ml streptomycin. Positive clones of *cdpA* complement strains were verified by PCR using the pUCP28T*cdpA* (ver) F and pUCP28T*cdpA* (ver) R primers showed both 2.5 kb *cdpA*::Tet and 375bp wild type *cdpA* PCR products. The restoration of the expression of *cdpA* was verified by RT-PCR using the primer pair: *cdpA* (RT-PCR) F and *cdpA* (RT-PCR) R to detect for the *cdpA* transcript.

Similarly, *BPSS0805* was amplified from *B. pseudomallei* KHW genomic DNA using PCR primer pairs: pUCP28T*BPSS0805* F and pUCP28T*BPSS0805* R. The PCR product was digested with BamHI, ligated to BamHI-linearised pUCP28T vector. The ligation product was transferred to *B. pseudomallei* KHW*BPSS0805*::Km mutant and wild type *B. pseudomallei* KHW via triparental conjugation to complement the KHW*BPSS0805*::Km mutation as well as to generate a *B. pseudomallei* KHW strain that overexpressed *BPSS0805*. Positive clones of

BPSS0805 complement strains verified by PCR using the pUCP28T*BPSS0805* (ver) F and pUCP28T*BPSS0805* (ver) R primers showed both 2.5 kb *BPSS0805*::Km and 711 bp wild type *BPSS0805* PCR products. The restoration of the expression of *BPSS0805* was verified by RT-PCR using the primer pair: *BPSS0805* (RT-PCR) F and *BPSS0805* (RT-PCR) R to detect for the *BPSS0805* transcript.

TABLE 2. Primers used in this study

Primer	Primer Sequence	Ann temp (^{0}C)
pJQ*cdpA*(BamHI)	F 5'- CGGGATCCGAAGCCATCAGGAACA -3' R 5'- ATGGATCCTCATGCGGTGGCGTG -3'	60
Tet (AccIII)	F 5'- GGCTCCGGACAAGGCGATTAA -3' R 5'- GCGTCCGGAGAATTAGCTTCAA -3'	57
cdpA::Tet(ver)	F 5'- ACAAGTTCGCGGTGATGCTG -3' R 5'- TCGTGATCGGCTGGAAATGC -3'	64
cdpA(RT-PCR)	F 5'- CGACGATTACCTGCGGATCAA- 3' R 5'- CGAGATAGTTGATGAGGCCGA- 3'	62
pUCP28T*cdpA*	F 5'- CCGGAATTCACGAGCGCGGTGAAGTCGAG -3' R 5'- CCGGAATTCACGTCAGCCCCTCGCCTGGA -3'	61
pJQ*BPSS0805* (BamHI)	F 5'- ATGGATCCAGGCGAGGCTCGAATAGC -3' R 5'- GCGGATCCCTACAACCTTTGGCTGGT -3'	65
Km (BsmI)	F 5'- GGGGAATGCGGAAAGGTTCCGTTCAGGACGCTA -3' R 5'- GGGGAATGCGGCCGAAGCCCAACCTTTCATA -3'	63
BPSS0805::Km(ver)	F 5'- CTCTTCACGGTCGCGATCCT -3' R 5'- GTCGCAGCGGAATTTCGGCT -3'	59
BPSS0805(RT-PCR)	F 5'- GATGAAGCCCGCCATCGAGT -3' R 5'- CGACGAAACGCTCTCCAGCA -3'	63
pUCP28T*BPSS0805*	F 5'- ATGGATCCAAGCCCTTCATGCAAACCCT -3' R 5'- GCGGATCCACCAACCGCAAACCCCCAAC-3'	60
pUCP28T*BPSS0805* (ver)	F 5'- TCCGGAGTATCCCTCGATCAAGGACTT -3' R 5'- TCCGGAGATGAAATCCAAGGGTTCCT -3'	61
16S rDNA	F 5' –GATGACGGTACCGGAAGAATAAGC-3' R 5' –CCATGTCAAGGGTAGGTAAGGTTT-3'	60

* Ann. Temp - annealing temperature used for PCR amplification.

2.6 Extraction of c-di-GMP from *B. pseudomallei* and its isogenic mutants

B. pseudomallei and its isogenic mutants were grown in AB media supplemented with 0.2% glucose and 0.5% casamino acids for 24 h until a cell density of OD_{600} ~1.8. Approximately 100 mg of wet weight of cells (equivalent to 9ml of OD_{600} ~1.8 culture) was harvested by centrifugation at 4000 g for 10 min. Nucleotides were extracted as previously described by Simm *et al.,* 2004. Briefly, the cells were first washed in 0.9% NaCl twice and heated at 100°C for 10 min. Next, the cell lysate was extracted twice with ice cold ethanol added to a final concentration of 65% (v/v). The extracts were lyophilized and resuspended in 200µl of water for subsequent HPLC analysis.

2.6.1 Reversed Phase High Performance Liquid Chromatography (RP-HPLC) analysis

The RP-HPLC analysis was performed with modifications based on Ryjenkov *et al,* 2005. Samples of 10 µl each were injected into a Hypersil C18 250x4.60mm column (Phenomenex, CA, USA) and separated by RP-HPLC (Agilent Series 1100). Elution of mixture was carried out at a flow rate of 0.7 ml/min with a gradient profile of Buffer A (100mM KH_2PO_4, 4 mM tetrabutyl ammonium hydrogen sulfate [pH 5.9] and buffer B (75% buffer A, 25% methanol). The conditions for reversed-phase HPLC are listed in Table 3.

Table 3. Running conditions of RP-HPLC for analysis of c-di-GMP

Time (min)	Percentage of Buffer B (%)
0 – 2.5	0
2.5 – 5	0 – 30
5 – 10	30 – 60
10 – 14	60 – 100
14 – 21	100
21 – 22	50
22 – 23	0

The peaks corresponding to the respective nucleotides were detected at 254 nm using Agilent 1100 Series variable wavelength detector and identified by their individual retention times (Appendix A). Calibration curves for GTP, GMP and c-di-GMP standards were obtained for identification and quantification purposes. Synthetic c-di-GMP was a kind gift from Associate Professor Lam Yulin, Department of Chemistry, National University of Singapore.

2.7 Phenotypic assays

2.7.1 Motility assay

Semi solid AB agar plates (0.3%) supplemented with 0.2% glucose (w/v) and 0.5% casamino acids were used to determine the motility of bacterial strains. The centre of the plates were inoculated with 2 µl overnight broth bacterial and incubated at 37°C for 24 h. Motility was assessed qualitatively by determining the circular swarm formed by the growing motile bacteria cells (Robleto *et. al.,* 2003).

2.7.2 Biofilm formation

Biofilm formation was assayed as per described previously by our laboratory (Chan and Chua, 2005). In brief, 100 µl of a diluted (OD600 of ~0.05) overnight bacterial culture in AB medium containing 0.2% glucose (w/v) and 0.5% casamino acids was added into each well of a 96-well microtiter plate. After 20 h of incubation at 30°C, the wells were washed twice with distilled water to remove planktonic cells. 125 µl of 1% (wt/vol) crystal violet (Sigma) was then added to the wells and incubated for 30 minutes at room temperature. After 30 minutes, the wells were washed thrice with 200 µl of distilled water and two aliquots of 150 µl of 95% (v/v) ethanol were added to solubilize the stain. The solubilized stain was then added to

400 ul water and the extent of biofilm formation was determined by the absorbance of the solution at 595 nm. The assay was performed in five independent wells for each bacteria strain.

2.7.3 Transmission electron microscopy

The overnight bacterial cultures of *B. pseudomallei* KHW, KHW*cdpA*::Tet mutant, its *trans* complementation and KHW/pUCP28T-*cdpA* strains were subcultured 1:50 into 5 ml of AB medium containing 0.2% glucose and 0.5% CAA. The bacteria were cultured to an optical density at 600 nm of 1.8 and then washed gently twice with 0.9%NaCl and fixed for 2 h in PBS containing 2.5% glutaraldehyde (Agar Scientific, Stansted, United Kingdom).

After a brief centrifugation and resuspension of the bacterial cells in PBS, a copper grid (Agar Scientific, Ltd., Essex, United Kingdom) was placed on a drop of bacterial suspension for 1 min. The grid was then dried and placed on a drop of a bacitracin solution (30 mg/ml; Sigma) for another 1 min and dried. The bacteria were negatively stained for 1 min by adding a drop of 1% phosphotungstate (pH 6.0; BDH, Poole, United Kingdom). The dried samples were examined with an EM208 S scanning electron microscope (Philips, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV with calibrated magnification.

2.7.4 Congo Red binding assay

Overnight bacterial cultures of *B. pseudomallei* KHW, KHW*cdpA*::Tet and KHW*BPSS0805*::Km mutants, the complemented mutants and KHW/pUCP28T-*BPSS0805* strains were streaked on LB agar plates without NaCl and supplemented with Congo Red (40 μg ml^{-1}) and Coomassie brilliant blue (20 μg ml^{-1}) (Römling *et al*, 1998). The plates were incubated for 24 h at 30°C and observed for extent of red

coloration. This experiment was carried in triplicates to ensure the consistency of the setup.

2.7.5 Cell aggregation assay

Cell aggregation studies were performed as described in Weber *et. al.,* 2006. Briefly, the bacteria were grown for 24 h at 37°C in 3ml of static LB medium. The cultures were then examined for cell aggregation and documented using photographs.

2.7.6 Cell invasion assay

The cell invasion assay was carried out as described previously by our laboratory (Chan *et. al*., 2007). A total of 1 x 10^5 per well of human lung carcinoma epithelial, A549 cells were infected with mid log phase (OD_{600} = 0.6) *B. pseudomallei* KHW, KHW*cdpA*::Tet and KHW*BPSS0805*::Km mutants, the complemented mutants and KHW/pUCP28T-*cdpA* and KHW/pUCP28T-*BPSS0805* strains separately at a MOI of 100:1. Two hours after infection, the A549 cells were centrifuged at 350 g for 3 min and supernatant was discarded. Subsequently, the A549 cells were washed twice with PBS, resuspended in medium containing 40 μg ml^{-1} tetracycline. After a further 2 h of incubation to kill extracellular bacteria, the wells were again washed thrice with phosphate buffered saline and 1 ml of 0.1% Triton X-100 (Sigma) was added to lyse the A549 cells. The cell lysate was subsequently diluted and then plated onto LA plates and incubated for 24 h to determine the number of bacteria in the cells after a 2 h exposure. The experiments were performed at least three times in triplicates.

2.7.7 Cytotoxicity assay

Cytotoxicity assay was carried out with slight modifications as described previously by our laboratory (Chan *et. al.,* 2007). A total of 2×10^6 cells/well were seeded in a 48-well plate in 0.6 ml of RMPI with 2% FCS and 2mM L-glutamine for 3 h before infection. Mid-log phase cultures (OD_{600}= 0.6) of *B. pseudomallei* KHW, KHW*cdpA*::Tet and KHW*BPSS0805*::Km mutants, the complemented mutants and KHW/pUCP28T-*cdpA* and KHW/pUCP28T-*BPSS0805* strains at approximately multiplicity of infection (moi) of 100:1 were added separately to the cells. Tetracycline (40 μg ml^{-1}) and kanamycin (200 μg ml^{-1}) were added 1 h after infection to suppress the growth of extracellular bacteria and further incubated for 4 h. The supernatant was then collected.

The cytotoxicity effect of the bacteria on mammalian cells were evaluated by the amount of lactate dehydrogenase released in the supernatant measured with Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instruction. Maximum release was achieved by lysis of cells with 1% Triton-X. LDH activity in supernatant of uninfected cells was taken as spontaneous release. Percentage cytotoxicity was calculated with the formula:

$$\% \text{ cytotoxicity} = \frac{(\text{Test LDH release} - \text{spontaneous release})}{(\text{Maximal release} - \text{spontaneous release})}$$

2.8 Statistical evaluation

The mean ± SD was calculated for each sample. Unless otherwise stated, all assays were performed in triplicate, and the mean was taken as 1 data point. Significant differences between means were determined by Analysis of Variance (ANOVA) post-hoc Tukey's multiple comparison tests (InStat, GraphPad software, San Diego, CA). P values of ≤ 0.05 were considered significant.

3.0 Results

3.1 Identification of putative GGDEF-EAL proteins in *B. pseudomallei*

Homology and conserved domain search identified a total of 16 genes encoding GGDEF-EAL domain proteins in the *B. pseudomallei* K96243 genome. As summarized in Table 4, five of these proteins contain both GGDEF – EAL domains while the remaining encodes either the GGDEF or EAL domain. Two other genes encoding HD-GYP domain proteins were also found in the genome. Like the EAL domain proteins, the HY-GYD domain proteins function as a phosphodiesterase and are involved in the hydrolysis of c-di-GMP (Ryan *et al.,* 2006).

Table 4. List of proteins containing GGDEF and/or EAL domain in *B. pseudomallei*

GGDEF-EAL domain	GGDEF domain	EAL domain	HD-GYP domain
BPSL 0602	BPSL 1306	BPSL 0358	BPSL0704
BPSL 1080	BPSS 0136	BPSL 0887	BPSS1648
BPSL 1263	BPSS 1297	BPSL 1286	
BPSS 0805	BPSS 1971	BPSL 1635	
BPSS 2318	BPSS 2342	BPSL 2744	
		BPSS 0799	

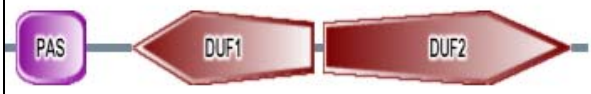
The genes investigated in this project are highlighted in bold.

In silico analysis of all 16 proteins using the Simple Modular Architecture Research Tool (SMART) showed that all 16 *B. pseudomallei* GGDEF-EAL proteins contained Domain of unknown Function with GGDEF motif (DUF 1) and / or Domain of unknown Function with EAL motif (DUF 2). In addition to the GGDEF / EAL domains, the SMART analysis showed that five of the *B. pseudomallei* proteins

are linked to a signal sensor domain (Table 5). These sensory domains, including the PAS, PAC and MHTY, are often found at the N terminus of proteins and are usually associated with signal sensing roles. Transmembrane segments were found in several of the GGDEF-EAL proteins, suggesting their location in the bacterial membrane. Collectively, the association of signal receiver domain and transmembrane segment with the GGDEF / EAL domains was consistent with the activities of these proteins in cell signalling and their regulation by environmental signals.

Table 5. Domain architecture of GGDEF-EAL proteins are predicted using Simple Modular Architecture Research Tool (SMART).

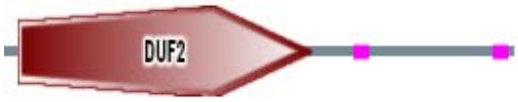
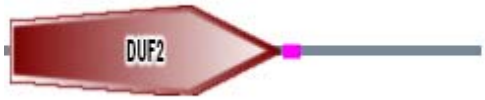
Putative *B. pseudomallei* proteins containing both GGDEF and EAL domains

BPSL 0602 Nucleotide positions: 676682 – 679045	
BPSL 1080 Nucleotide positions: 1249558 – 1251918	
BPSL 1263 **Nucleotide positions: 1458136 – 1459878** **(*cdpA*)**	
BPSS 0805 **Nucleotide positions: 1077778 – 1079844**	
BPSS 2318 Nucleotide positions: 3119667 - 3122108	

Putative *B. pseudomallei* proteins containing only the GGDEF domain

BPSL 1306 Nucleotide positions:1524128 - 1525492	
BPSS 0136 Nucleotide positions: 175204 - 176592	
BPSS 1297 Nucleotide positions: 1776176 - 1777645	
BPSS 1971 Nucleotide positions: 2662056 - 2663579	
BPSS 2342 Nucleotide positions: 3158698 - 3159633	

Putative *B. pseudomallei* proteins containing only EAL domain

BPSL 0358 Nucleotide positions: 385324 - 386613	
BPSL 0887 Nucleotide positions: 1028626 - 1029900	
BPSL 1286 Nucleotide positions: 1497670 - 1498881	
BPSL 1635 Nucleotide positions: 1899405 - 1900298	
BPSL 2744 Nucleotide positions: 3286594 - 3287400	
BPSS 0799 Nucleotide positions: 1071034 – 1072254	

Legend

DUF 1 – Domain of Unknown Function with GGDEF motif
DUF 2 – Domain of Unknown Function with EAL motif
- Transmembrane segment
- Signal peptide
PAS – A common signal sensor domain, the PAS domain was named after three proteins that it occurs in Drosophila period clock protein (**P**ER), vertebrate aryl hydrocarbon receptor nuclear translocator (**A**RNT), and Drosophila single-minded protein (**S**IM)
PAC – A C-terminal motif to the PAS motifs and is proposed to contribute to the PAS domain fold.
MHYT – Named after its conserved amino acid motif, methionine, histdine and tyrosine. This domain is thought to function as a sensor domain in bacterial signaling proteins.
BPSL – Genes annotated as BPSL are located on *B.pseudomallei* chromosome 1
BPSS – Genes annonated as BPSS are located on *B. pseudomallei* chromosome 2

3.2 *In silico* analysis of *BPSL1263*

From the list of *B. pseudomallei* GGDEF-EAL proteins in Table 5, two proteins, BPSL1263 and BPSS0805 were selected for further investigations in this study. The physical map of *BPSL1263,* which was subsequently annotated as c-di-GMP phosphodiesterase A (*cdpA*) after its phosphodiesterase activity, is illustrated in Fig. 1A. Located on chromosome one of *B. pseudomallei* from nucleotide positions, 1458136 to 1459878, *cdpA* is 1743 bp in length and predicted to encode a polypeptide of 581 amino acids (63.9 kDa). Using the Simple Modular Architecture Research Tool, the GGDEF domain of CdpA was predicted to span 182 amino acids from a.a. positions 127 to 308 (E-value of 1.1e-34) and its EAL domain spanned 247 a.a. from positions 318 to 564 (E-value of 5.9e-115). In addition, a N terminal PAS domain was identified at amino acids positions 12 to 81. Using TMpred tool, a transmembrane spanning region at amino acids positions 361 to 386 was identified.

Pair-wise comparison of BPSL1263 with several other previously investigated c-di-GMP PDE proteins revealed that the *B. pseudomallei* BPSL1263 shares ~30% amino acid sequence identity (Table 6). Interestingly several proteins of these proteins such as *P. aeruginosa* PA2567 (GenBank accession no: NP_251257), PA5017 (GenBank accession no: 253704), *C. crescentus* CC3366 (GenBank accession no: NP_422190) and *Y. pestis* Y3832 (GenBank accession no: NP_671126) also did not carry the conserved GG(D/E)EF domain, suggesting these too do not encode functional DGC activities as was the case for BPSL1263.

3.3 *In silico* analysis of BPSS0805

The physical map of the second GGDEF-EAL protein, *BPSS0805* was illustrated in Fig. 1B. Located at nucleotide positions, 1077778 – 1079844 on the *B. pseudomallei* chromosome two, the gene was predicted to encode a membrane associated protein of 688 amino acids, with a molecular weight of 74.4 kDa. Using Simple Modular Architecture Research Tool, the GGDEF domain of BPSS0805 is located at amino acids positions 246 to 418 (173 amino acids; E-value of 1.5e-65) and its EAL domain from amino acids positions 428 to 675 (248 amino acids; E-value of 7.10e-98). Two MHYT domains were located at the N terminal of the protein at positions 52 to 113 and 115 to 177 respectively. Using TMpred tool, eight transmembrane helices, at amino acid positions 7 to 25; 42 to 64; 78 to 98; 107 to 125; 140 to 160; 174 to 192; 213 to 233 and 622 to 640 were identified.

BBSS0805 was also found to exhibit a high level of homology with several other well studied GGDEF-EAL domain proteins (Table 7). Notably, BPSS0805 share 44% amino acid sequence identity to motility regulator of *P. aeruginosa,* MorA (GenBank accession no: NP_253291), which led to investigations on whether BPSS0805 shared similar properties as MorA with regards to c-di-GMP metabolism, regulation of motility and formation of biofilm in *B. pseudomallei.*

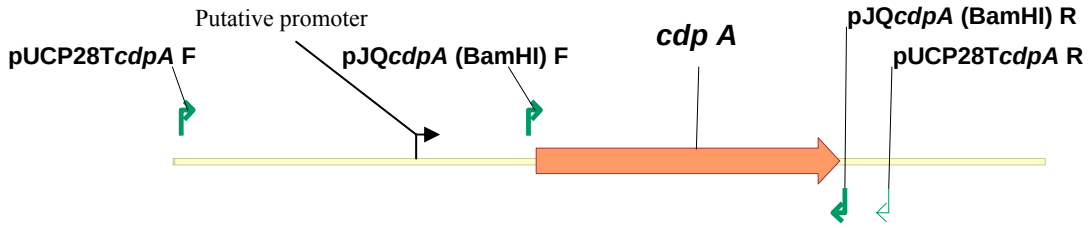


Fig. 4A. Physical map of the *B. pseudomallei cdpA* gene.
The location of *cdpA* is on chromosome one from position 1458136 to 1459878. Orange arrows denote codons with the direction of transcription indicated by the direction of the arrowhead. The names of the primers used to amplify *cdpA* are shown as green. The black arrow indicates the location of the putative promoter predicted using the Neural Network Promoter Prediction (NNPP) program for prokaryotes.

Table 6: Comparison of percentage of identity between *B. pseudomallei* BPSL1263 and selected c-di-GMP PDE homologues

Microbes (Protein Id / GenBank Accession No.)	% identity to *B. pseudomallei* BPSL1263	Reference
V. cholera (VieA / NP_231289)	35%	Tamayo *et. al.,* 2005
C. crescentus (CC3366 / NP_422190)	35%	Christen *et. al.,* 2005
P. aeruginosa (PA5017 / NP_253704)	35%	Li *et. al.,* 2007
E. coli (YahA / NP_414849)	34%	Schmidt *et. al.,* 2005
Y. pestis (HmsP / NP_671126)	33%	Kirillina *et. al.,* 2004
P. aeruginosa (PA2567 / NP_251257)	33%	Ryan *et. al.,* 2006

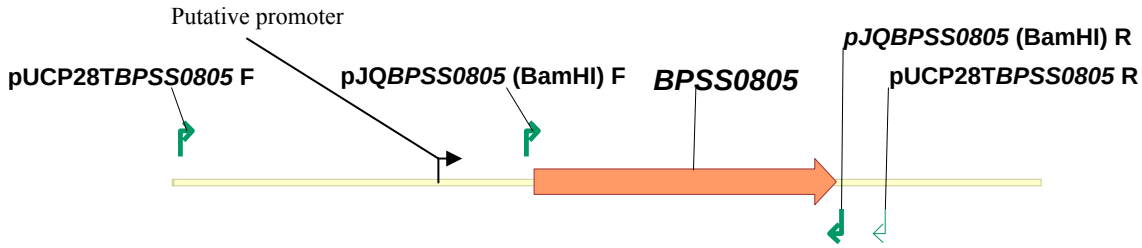


Fig. 4B. Physical map of the *B. pseudomallei BPSS0805* gene.
The location of *BPSS0805* is on chromosome two from position 1077778 – 1079844. Orange arrows denote codon with the direction of transcription indicated by the direction of the arrowhead. The names of the primers used to amplify *BPSS0805* are shown as green. The black arrow indicates the location of the putative promoter predicted using the Neural Network Promoter Prediction (NNPP) program for prokaryotes.

Table 7: Comparison of percentage of identity between *B. pseudomallei* BPSS0805 and selected homologues

Microbes (Protein Id / GenBank Accession No.)	% identity to *B. pseudomallei* BPSS0805	Reference
P. aeruginosa (MorA / NP_253291)	44%	Choy *et. al*., 2004
C. crescentus (PleD / NP_421265)	38%	Aldridge *et al.* 2003
Y.pestis (HmsT / NP_671050)	33%	Kirillina *et. al.,* 2004
P.aeruginosa (WspR NP_252391)	32%	D'Argenio *et al.,* 2002
E. coli (YdaM / NP_415857)	34%	Weber *et. al.,* 2006
V. cholerae (VCA0956 / NP_233340)	30%	Tischler and Camilli, 2004
S. typimurium (AdrA / NP_454980)	27%	Romling *et. al.,* 2002

3.4 Mutagenesis and complementation

3.4.1 Construction of the isogenic KHW*cdpA*::Tet mutant

Targeted insertional mutagenesis of *cdpA* was employed to create *cdpA* null mutant of the virulent clinical strain, *B. pseudomallei* KHW. The suicide vector, pJQ200mp18, harboring a sucrose-inducible lethal gene, *sacB*, allowed the selection of clones that had undergone gene replacement on media containing sucrose and the antibiotic resistance cassette used for disrupting the *cdpA* and *BPSS0805* (Quandt and Hynes, 1993).

An isogenic derivative of wild type *B. pseudomallei* KHW containing an insertion mutation in *cdpA* was constructed using the suicide vector pJQ200mp18. The 1743-bp full-length gene was amplified from *B. pseudomallei* KHW genomic DNA and disrupted by inserting a Tet^R cassette at the *Acc*III restriction site within *BPSL1263*. Restriction digestion of pJQ200mp18*cdpA*::Tet plasmid with *Bam*H1 showed two distinct bands, corresponding to the pJQ200mp18*cdpA* and Tet^R cassette (data not shown). The insertion of the Tet^R cassette into *cdpA* was confirmed by PCR using primer pair: Tet (*Acc*III) F and Tet (*Acc*III) F, which yielded a 2.1 kb fragment that corresponded to the size of the Tet^R cassette (Fig. 5, Lane 1), as well as by DNA sequencing using primer pair: *cdpA*::Tet(ver) F and *cdpA*::Tet(ver) R (data not shown).

The plasmid was then introduced into *B. pseudomallei* KHW by triparental mating and exconjugants were selected on 200 μg/ml tetracycline, 100 μg/ml streptomycin and 5% sucrose medium. The vector pJQ200mp18, which carried a sucrose-inducible lethal gene, *sacB*, facilitated the selection of double crossover recombinants carrying the insertional mutation on the chromosome by allelic exchange (Quandt and Hynes, 1993). To verify the insertional mutagenesis of *cdpA*, it

is important to demonstrate that the *cdpA* was indeed disrupted by the Tet^R cassette. Our PCR results using primer pair: Tet (*Acc*III) F and Tet (*Acc*III) R showed that a 2.1 kb fragment was amplified from pJQ200mp18*cdpA*::Tet plasmid and *B. pseudomallei* KHW*cdpA*::Tet genomic DNA (Fig. 5, Lane 1 and 3) but no PCR product was amplified from *B. pseudomallei* KHW genomic DNA (negative control) (Fig. 5, Lane 2). Furthermore, PCR with *cdpA*::Tet(ver) primers yielded only a 375 bp fragment from *B. pseudomallei* KHW genomic DNA while a 2500 bp from KHW*cdpA*::Tet genomic DNA (Fig. 6, Lane 1 and 2).

Disruption of *cdpA* was verified by RT-PCR to confirm absence of *cdpA* gene expression.The null mutation in the *B. pseudomallei* KHW*cdpA*::Tet mutant was verified by checking for the presence of *cdpA* mRNA. RT-PCR, using *cdpA*(RT-PCR) primers, on total RNA isolated from KHW*cdpA*::Tet mutant and the parental strain *B. pseudomallei* KHW, was carried out. The 626 bp *cdp*A transcript was detected in the wild-type *B. pseudomallei* KHW parental strain but not in the KHW*cdpA*::Tet mutant, thus confirming the successful disruption of *cdpA* expression (Fig. 7, Lanes 1 and 3). RT-PCR of 16S rRNA was included as an internal control. The absence of amplified band in lanes without reverse transcriptase (RT) showed no contaminating DNA in the RNA preparations (Fig. 7, Lanes 2, 4, 6 and 8).

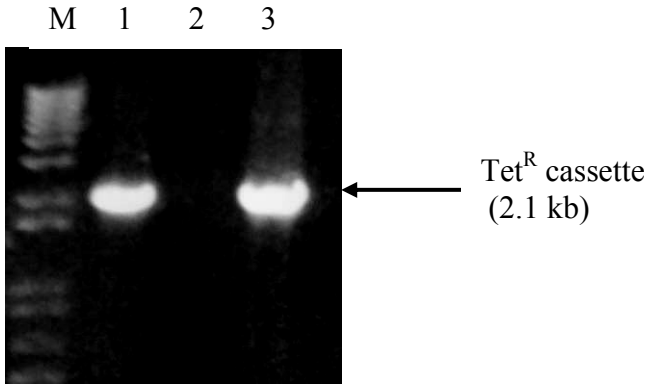


Fig. 5. PCR verification of Tet^R in pJQ200mp18*cdpA*::Tet plasmid and KHW*cdpA*::Tet null mutant.
PCR amplifications were done on pJQ200mp18*cdpA*::Tet plasmid (Lane 1), wild type *B. pseudomallei* KHW genomic DNA (Lane 2) and KHW*cdpA*::Tet mutant genomic DNA (Lane 3) using primer pairs Tet(AccIII) F and Tet(AccIII) R. Lane M: 1kb plus DNA marker

3.4.2 Construction of KHW*cdpA*::Tet/pUCP28T-*cdpA* and KHW/pUCP28T-*cdpA*

Complementation of the *cdpA* mutation by introducing a pUCP28T plasmid harboring the full length *cdpA* gene into the mutant via triparental conjugation was necessary for confirmation that the phenotypes associated with *B. pseudomallei* KHW*cdpA*::Tet mutant were indeed due to the loss of *cdpA* gene function and not a secondary site or polar mutation. Successful introduction of the pUCP28T-*cdpA* plasmid in KHW*cdpA*::Tet mutant was confirmed via PCR using *cdpA*::Tet(ver) primers. Two distinct PCR fragments, a 375 bp fragment amplified from the undisrupted *cdpA* on the pUCP28T vector and a 2500 bp fragment yielded from the Tet^R inserted chromosomal *cdpA* was clearly observed (Fig. 6, Lane 3).

The complemented KHW*cdpA*::Tet mutant was shown to expresss the cdpA transcript by RT-PCR using *cdpA*(RT-PCR) primers (Fig. 7, Lane 5). *B. pseudomallei* KHW overexpressing *cdpA* was generated by introducing the pUCP28T-*cdpA* plasmid into wild-type cells. Successful introduction of pUCP28T-*cdpA* plasmid in

the wild type KHW was verified by PCR (Fig. 7, Lane 4) and detection of *cdpA* expression by RT-PCR using *cdpA* (RT-PCR) primers (Fig. 7, Lane 7).

However, due to the semi quantitative nature of reverse transcription PCR, the results did not illustrate the overexpression of *cdpA* in the KHW/pUCP28T-*cdpA* strains. For future studies, quantitative real time PCR experiments should be carried out to quantitatively determine the expression levels of *cdpA* in the constructed mutants and their respective complement strains.

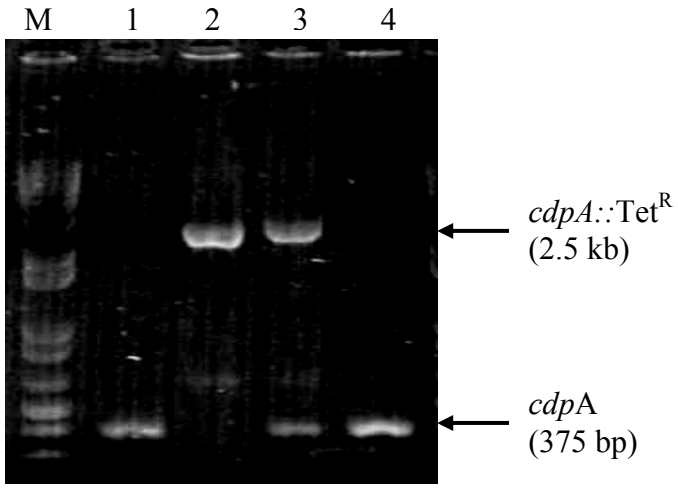


Fig. 6. PCR verification of KHW*cdpA*::Tet mutant, *cdpA* complement and KHW/pUCP28T-*cdpA* in *B. pseudomallei.*
PCR amplifications were done on wild type *B. pseudomallei* KHW genomic DNA (Lane 1), KHW*cdpA*::Tet (Lane 2), KHW*cdpA*::Tet/pUCP28T-*cdpA* (Lane 3) and KHW/pUCP28T-*cdpA* (Lane 4) using primer pairs *cdpA*::Tet(ver) F and *cdpA*::Tet(ver) R. Lane M: 1kb plus DNA marker

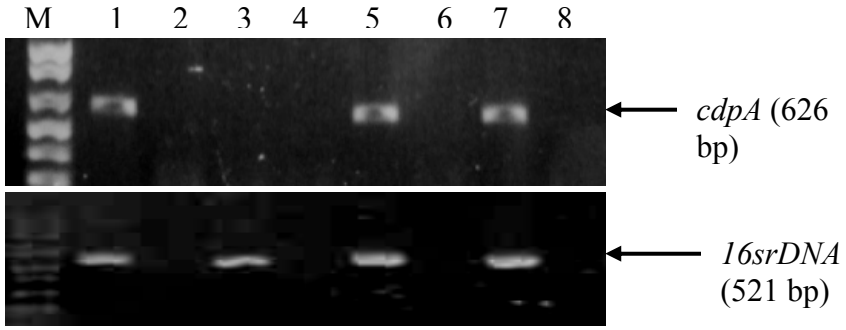


Fig. 7. Detection of *cdpA* expression in wild type *B. pseudomallei* KHW, KHW*cdpA*::Tet, *cdpA* complement and KHW/pUCP28T-*cdpA* by RT-PCR.
120 ng of total RNA isolated from KHW (Lane 1 and 2) and its isogenic mutant KHW*cdpA*::Tet (Lane 3 and 4), KHW*cdpA*::Tet/pUCP28T-*cdpA* complement strain (Lane 5 and 6) and KHW/pUCP28T-*cdpA* (Lane 7 and 8) were used for RT-PCR to detect *cdpA* expression.
The presence of *cdpA* transcript in lane 1 but not lane 3 indicated successful knockout of *cdpA*. In lane 5, the presence of a bright band corresponding to the size of *cdpA* transcript indicated the successful complementation of *cdpA*. The absence of any complementary DNA (cDNA) bands in RT-PCR reactions without reverse transcriptase (Lanes 2, 4, 6 and 8) indicated absence of DNA contamination in the RNA samples. RT-PCR results of 16S *rDNA* using primer pairs 16S rDNA F and 16S rDNA R were included as an internal control.

3.4.3 Construction of isogenic KHW*BPSS0805*::Km mutant

Using methods similar to the construction of KHW*cdpA*::Tet mutant, an isogenic *BPSS0805* mutant was constructed. For this mutation, a Km^R cassette was used to disrupt the gene. Restriction digestion with BsmI was performed to verify the constructed pJQ200mp18*BPSS0805*::Km plasmid (Data not shown). Further confirmation with PCR using primer pair: Km(*Bsm*I) F and Km(*Bsm*I) R on the plasmid yielded a 1.8 kb fragment that corresponded to the size of the Km^R cassette (Fig. 8, Lane 1). The pJQ200mp18*BPSS0805*::Km plasmid was then successfully introduced into *B. pseudomallei* KHW and selected on 200 µg/ml kanamycin, 100 µg/ml streptomycin and 5% sucrose medium. Similar to the verification of KHW*cdpA*::Tet mutant, the insertion of the kanamycin-resistance cassette into *BPSS0805* was also verified by PCR using Km(BsmI) primers pairs. A 1.8 kb fragment amplified from KHW*BPSS0805*::Km but not from wild type *B. pseudomallei* KHW (Fig. 8, Lanes 2 and 3). Subsequent PCR amplification of the KHW*BPSS0805*::Km genomic DNA with BPSS0805::Km(ver) F and BPSS0805::Km(ver) R primers produced a 2542 bp PCR product as compared to a 711 bp PCR product from wild type *B. pseudomallei* KHW genomic DNA (Fig. 9, Lanes 1 and 2).

The null mutation in the *B. pseudomallei* KHW*BPSS0805*::Km mutant was verified by absence of *BPSS0805* transcript by RT-PCR of total RNA prepared from KHW*BPSS0805*::Km mutant using *BPSS0805* (RT-PCR) primers. The 658 bp BPSS0805 transcript was detected in the wild-type *B. pseudomallei* KHW parental strain but not in the KHW*BPSS0805*::Km mutant, thus confirming the successful abolition of *BPSS0805* gene expression (Fig. 10, Lanes 1 and 3). RT-PCR of 16S rRNA was included as an internal control. The absence of amplified band in lanes

without reverse transcriptase (RT) showed an absence of contaminating DNA in the RNA preparations (Fig. 10, Lanes 2, 4, 6 and 8).

3.4.4 Construction of *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805*

pUCP28T vector harboring the full length *BPSS0805* gene was used to complement the *BPSS0805* expression in KHW*BPSS0805*::Km, as well as to generated wild-type cells carrying multiple copies of *BPSS0805.* The presence of the pUCP28T-*BPSS0805* plasmid in KHW*BPSS0805*::Km mutant was confirmed by PCR using BPSS0805::Km (ver) F and BPSS0805::Km (ver) F primer. Two distinct PCR fragments, a 658 bp fragment amplified from the full length *BPSS0805* on the pUCP28T vector and a 2489 bp fragment yielded from the disrupted chromosomal *BPSS0805* was clearly observed (Fig. 9, Lane 3). Successful complementation of the KHW*BPSS0805*::Km mutant was demonstrated by RT-PCR using BPSS0805 (RT-PCR) primers which detected the *BPSS0805* transcript (Fig. 10, Lane 5). Successful introduction of the pUCP28T-*BPSS0805* plasmid in the *B. pseudomallei* KHW was also verified by PCR (Fig. 9, Lane 4) and confirmed by RT-PCR using BPSS0805 (RT-PCR) F and BPSS0805 (RT-PCR) R primers (Fig. 10, Lane 7).

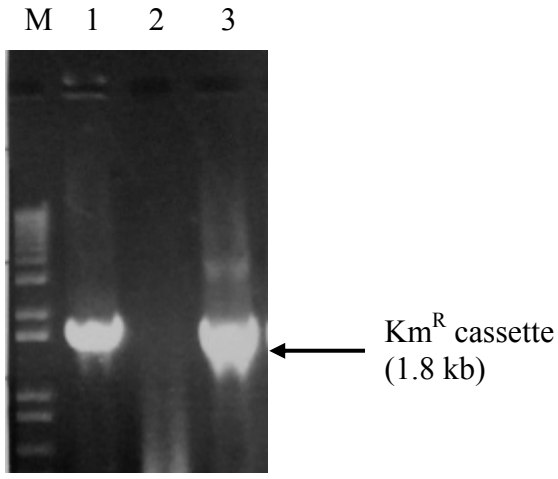


Fig. 8. PCR verification of Km^R in pJQ200mp18-*BPSS0805*::Km plasmid and KHW*BPSS0805*::Km null mutant.
PCR amplifications were done on pJQ200mp18*BPSS0805*::Km plasmid (Lane 1), wild type *B. pseudomallei* KHW genomic DNA (Lane 2) and KHW*BPSS0805*::Km mutant genomic DNA (Lane 3) using primer pairs Km(BsmI) F and Km(BsmI) R. Lane M: 1kb plus DNA marker

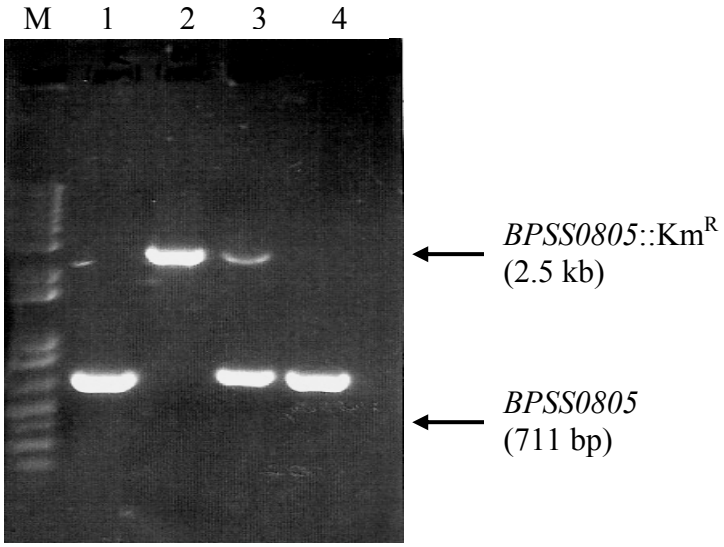


Fig. 9. PCR verification of KHW*BPSS0805*::Km mutant, KHW*BPSS0805*::Km/ pUCP28T-*BPSS0805* and KHW/pUCP28T-*BPSS0805* in *B. pseudomallei.*
PCR amplifications were done on wild type *B. pseudomallei* KHW genomic DNA (Lane 1), KHW*BPSS0805*::Km (Lane 2), KHW*BPSS0805*::Km/pUCP28T-*BPSS0805* (Lane 3) and KHW/pUCP28T-*BPSS0805* (Lane 4) using primer pairs BPSS0805::Km (ver) F and BPSS0805::Km (ver) R. Lane M: 1kb plus DNA marker

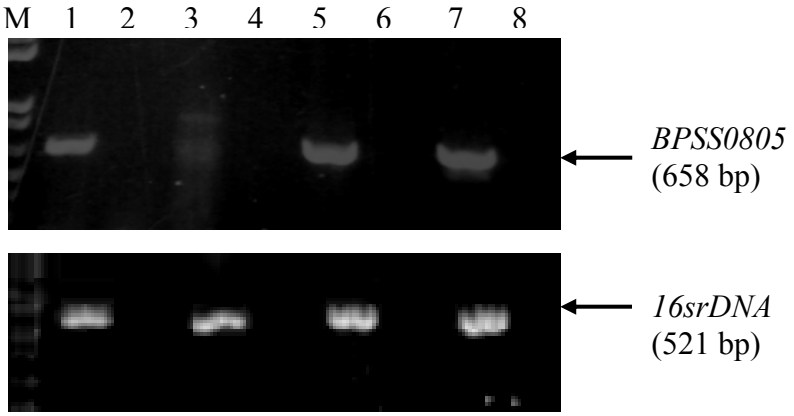


Fig. 10. Detection of *BPSS0805* expression in *B. pseudomallei* KHW and its isogenic mutant, KHW*BPSS0805*::Km, KHW*BPSS0805*::Km/pUCP28T-*BPSS0805* and KHW/ pUCP28T-*BPSS0805* by RT-PCR.

120 ng of total RNA isolated from *B. pseudomallei* KHW (Lane 1 and 2) and its isogenic mutant KHW*BPSS0805*::Km (Lane 3 and 4), KHW*BPSS0805*::Km/pUCP28T*BPSS0805* complemented mutant (Lane 5 and 6) and KHW/pUCP28T*BPSS0805* (Lane 7 and 8) were used for RT-PCR to detect *BPSS0805* expression.

The presence of *BPSS0805* transcript in lane 1 but not lane 3 indicates successful *BPSS0805* null mutation. In lane 5, the presence of a bright band corresponding to the size of *BPSS0805* transcript indicates the successful complementation of *BPSS0805*. The absence of any complementary DNA (cDNA) bands in RT-PCR reactions with reverse transcriptase (Lanes 2, 4, 6 and 8) indicated absence of DNA contamination in the RNA samples. RT-PCR of 16*SrDNA* using primer pairs 16SF2 and 16SR2 were included as an internal control.

3.5 The *in vivo* functional characterization of CdpA and BPSS0805

3.5.1 CdpA affects the intracellular c-di-GMP level *of B.pseudomallei*

Based on the predicted involvement of GGDEF and EAL domain in c-di-GMP turnover, the presence of intracellular c-di-GMP was analyzed to determine the role of CdpA *in vivo.* The HPLC analysis of the extracted nucleotides showed that the basal level of c-di-GMP in stationary phase wild type *B. pseudomallei* KHW was in the range of 5.58 +/- 0.69 pmol of c-di-GMP per mg wet weight (Fig. 11). In contrast, the deletion of *cdpA* resulted in a marked increase in the levels of c-di-GMP in the KHW*cdpA*::Tet mutant. An eight fold increase in intracellular level of c-di-GMP (40.73 +/- 3.22 pmol mg^{-1} wet weight) was detected in KHW*cdpA*::Tet compared to wild type *B. pseudomallei* KHW (Fig. 11). A plausible explanation for the higher intracellular level of c-di-GMP in the *cdpA* mutant is a reduced phosphodiesterase activity in the mutant, therefore implicating CdpA's role as a c-di-GMP phosphodiesterase in *B. pseudomallei*.

The implication of CdpA as a phosphodiesterase was also verified by examining the intracellular c-di-GMP content of the *trans* complemented *cdpA*. The results showed that the *cdpA* complemented mutant contained 5.37 +/- 0.78 pmol of intracellular c-di-GMP per mg wet weight. Using ANOVA analysis with post-hoc Tukey's multiple comparison test, no statistically significant difference was found in the levels of c-di-GMP between the *cdpA* complemented mutant and wild type *B. pseudomallei* KHW ($P>0.05$), thus demonstrating successful complementation of the *cdpA* mutation by introducing the pUCP28T-*cdpA* plasmid.

In *B. pseudomallei* KHW/pUCP28T-*cdpA*, the amount of c-di-GMP detected was 3.26 +/- 0.16 pmol / mg wet cell weight, which is 40 % lower than the intracellular levels of c-di-GMP detected in wild type *B. pseudomallei* KHW and

cdpA complemented mutant (P<0.05), thus verifying that *cdpA* affects the intracellular content of c-di-GMP of *B. pseudomallei* (Fig. 11).

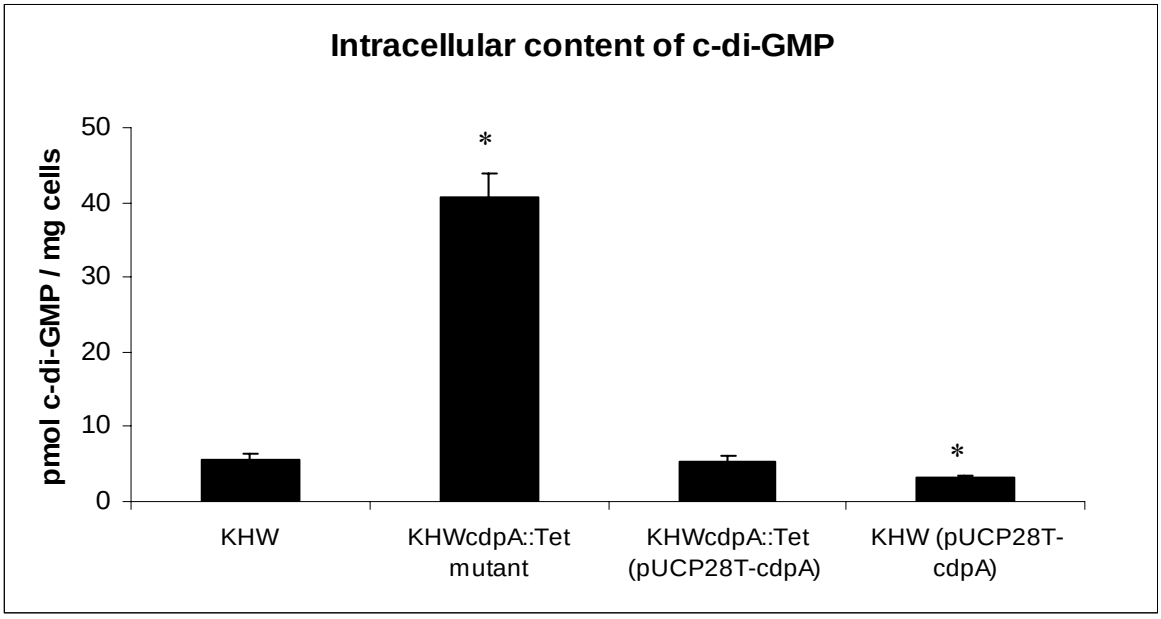


Fig. 11. Intracellular content of c-di-GMP of wildtype *B. pseudomallei* KHW, KHW*cdpA*::Tet, KHW*cdpA*::Tet/pUCP28T-*cdpA* and KHW/pUCP28T-*cdpA*
Intracellular nucleotides in late stationary phase cells were extracted and analyzed by RP-HPLC for c-di-GMP. The strains assayed included wild type *B. pseudomallei* KHW, KHW*cdpA*::Tet mutant, KHW*cdpA*::Tet/pUCP28T-*cdpA* complement strain and KHW/pUCP28T-*cdpA*. The amount of c-di-GMP is expressed per milligram (wet weight) of cells. The amount of c-di-GMP in wild type *B. pseudomallei* KHW is in the range of 5 pmol mg^{-1} of wet weight of cells.
In contrast, null mutation of *cdpA* resulted in an eightfold increased in the amount of c-di-GMP. Complementation of *cdpA* restored the c-di-GMP levels back to the wild type levels. The amount of intracellular c-di-GMP was further decreased in KHW/pUCP28T-*cdpA* to 3.26 +/- 0.16 pmol / mg of wet weight of cells. Each bar is a mean of three independent experimental determinations. An asterisk denotes a statistically significant difference ($P < 0.05$) from the values of wild type *B. pseudomallei* KHW as judged by the ANOVA test.

3.5.2 Intracellular c-di-GMP levels were not altered in the BPSS0805 null mutant

To investigate the role of *in vivo* functional role BPSS0805 in the turnover of c-di-GMP, the levels of intracellular c-di-GMP in *BPSS0805* null mutant, its complemented strain and KHW/pUCP28T-*BPSS0805* were examined. The results showed that the amount of c-di-GMP in the KHW*BPSS0805*::Km mutant and *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805* were all in the range of 5pmol of c-di-GMP per mg of wet weight of cells (Fig. 12). Using ANOVA analysis with post-hoc Tukey's multiple comparison test, no significant difference was found between the strains. (P> 0.05)

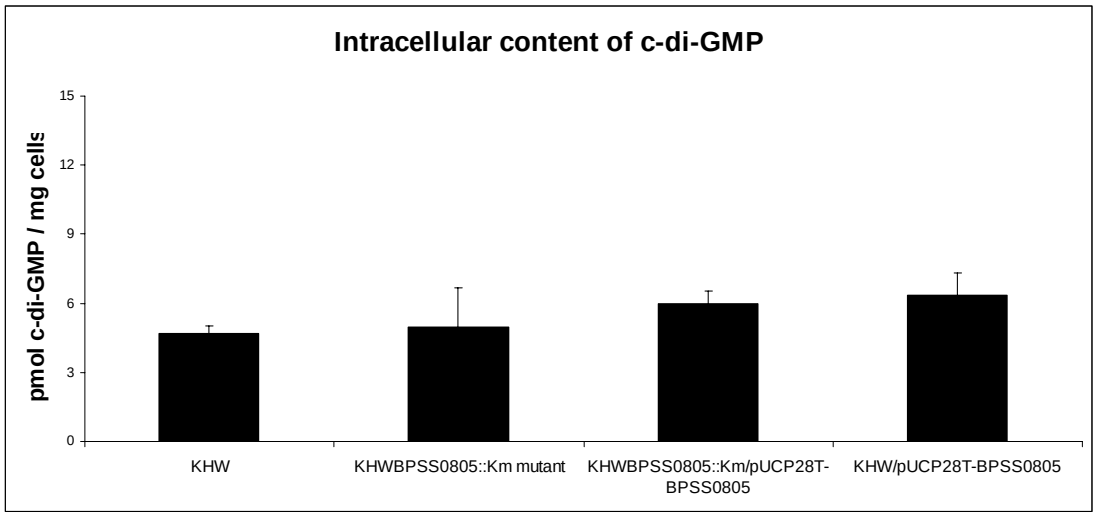


Fig. 12. Intracellular content of c-di-GMP of wildtype *B. pseudomallei* KHW, KHW*BPSS0805*::Km, *BPSS0805* complement and KHW/pUCP28T-*BPSS0805*. No significant difference in the amount of intracellular c-di-GMP between the wild type *B. pseudomallei* KHW and KHW*BPSS0805*::Km mutant, *BPSS0805* complement and KHW/pUCP28T-*BPSS0805* was detected. Each bar is a mean of three independent experimental determinations. Using ANOVA analysis with post-hoc Tukey's multiple comparison test, no significant difference was found between the strains. (P> 0.05)

3.6 Phenotypic assays of the *cdpA* and *BPSS0805* null mutants

As reviewed in Section 1, GGDEF-EAL proteins in different bacteria regulate diverse bacterial properties. In this study, the roles in *B. pseudomallei* CdpA and BPSS0805 in motility, flagellar development, cell aggregation, cellulose synthesis, biofilm formation and virulence were investigated.

3.6.1 CdpA, but not BPSS0805, is required for swimming motility in *B. pseudomallei*

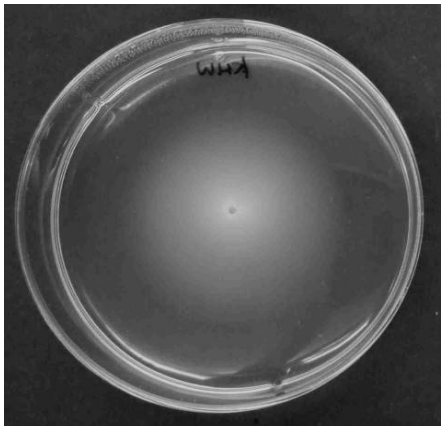
The correlation between c-di-GMP and bacterial motility has been well documented in several bacteria including *E. coli, S. typimurium, P. aeruginosa.* High levels of intracellular c-di-GMP levels were found to inhibit motility while conversely, low levels of c-di-GMP promotes motility (reviewed by Tamayo *et. al.,* 2007).

As the previous results have shown that a higher intracellular level of c-di-GMP was detected in KHW*cdpA*::Tet mutant relative to wild type *B. pseudomallei* KHW, it is proposed that the higher level of intracellular level of c-di-GMP in the mutant could possibly lead to a decrease in motility. To this end, the swimming motility of the KHW*cdpA*::Tet mutant was tested in 0.3% (wt/vol) semisolid AB agar, supplemented with 0.2% glucose and 0.5% casamino acids. After incubation at 37ºC for 24 hrs, the mutant showed a significant reduction in swimming motility compared to wild type *B. pseudomallei* KHW (Fig. 13).

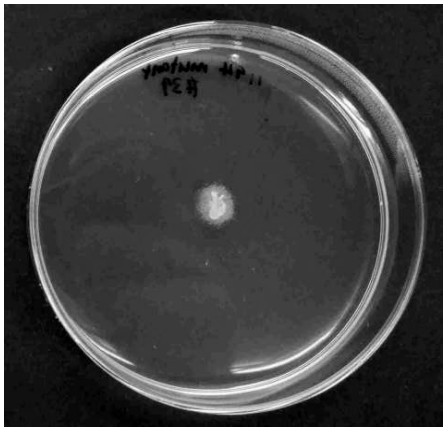
To ascertain if the swimming motility defect was a result of *cdpA* mutation rather than a secondary or polar mutation, the swimming motility of *cdpA trans* complemented strain was similarly tested. The results showed that a complete restoration of swimming motility by the complementation of *cdpA***.** There was also a

slight increase in the swimming motility of KHW/pUCP28T-*cdpA* relative to wild type *B. pseudomallei* KHW (Fig. 13).

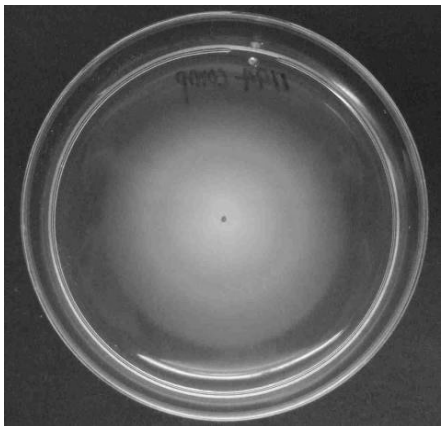
As shown in Section 3.5.2, there was no significant difference in the level of intracellular c-di-GMP between wild type *B. pseudomallei* KHW and *BPSS0805* mutant. Hence, the swimming motility of *BPSS0805* mutant, its complement and KHW/pUCP28T-*BPSS0805* strain was investigated to correlate the level of intracellular c-di-GMP with bacterial swimming motility. Not surprisingly, no observable difference was detected in the motility between wild type *B. pseudomallei* KHW and *BPSS0805* null mutant, thereby suggesting that *BPSS0805* mutation, which did not affect intracellular c-di-GMP, also did not affect bacterial swimming motility. In addition, the swimming motility of its complemented mutant and KHW/pUCP28T-*BPSS0805* were also shown to be similar to wild type *B. pseudomallei* KHW. Taken together, these findings further support the correlation between intracellular c-di-GMP levels and bacterial swimming motility and possibly functional redundancy of BPSS0805 *in vivo* (Fig. 14).



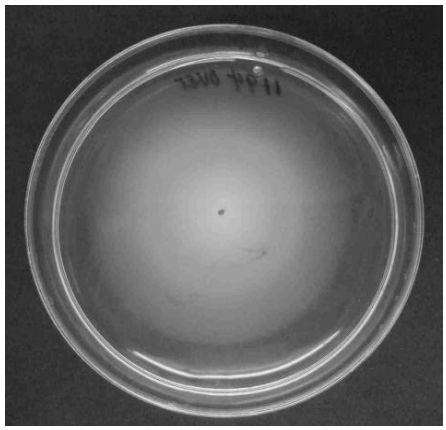
B. pseudomallei KHW



KHW*cdpA*::Tet mutant



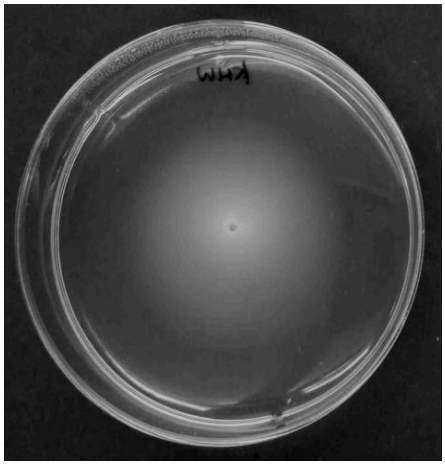
KHW*cdpA*::Tet/pUCP28T-*cdpA*



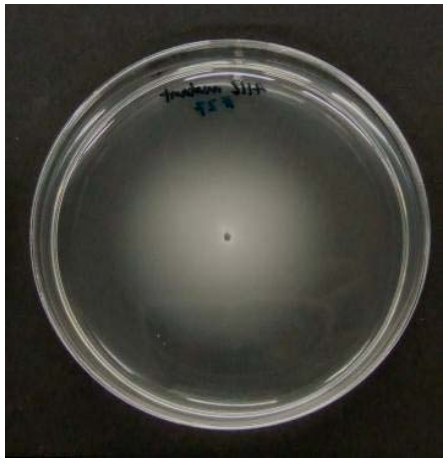
KHW/pUCP28T-*cdpA*

Fig. 13. Swimming motility of wild type *B. pseudomallei* KHW, KHW*cdpA*::Tet mutant, KHW*cdpA*::Tet/pUCP28T-*cdpA* and KHW/pUCP28T-*cdpA* in semisolid agar.

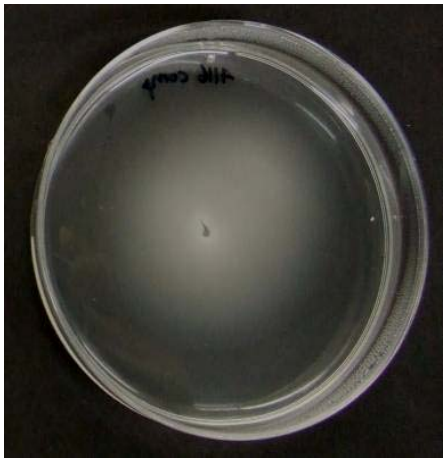
Motility of KHW*cdpA*::Tet mutant was significantly inhibited relative to wild type *B. pseudomallei* KHW in semisolid AB agar (0.3% wt/vol). This motility defect was restored by the *trans* complementation of the *cdpA* in the mutant. The KHW/pUCP28T-*cdpA* exhibit a small increase in swimming motility phenotype relative to the parental wild type *B. pseudomallei* KHW.



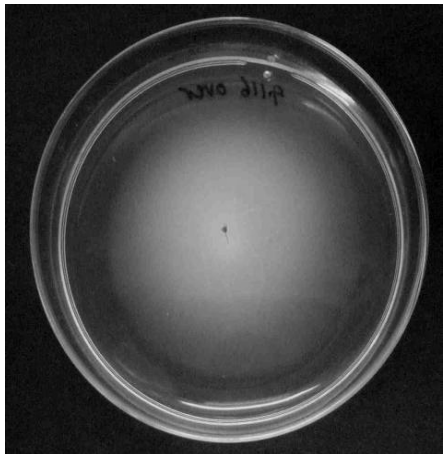
B. pseudomallei KHW



KHW*BPSS0805*::Km mutant



KHW*BPSS0805*::Km/pUCP28T-*BPSS0805*



KHW/pUCP28T-*BPSS0805*

Fig. 14. Swimming motility of wild type *B. pseudomallei* KHW was not affected by *BPSS0805* null mutation.
Swimming motility of KHW*BPSS0805*::Km mutant was similar to wild type *B. pseudomallei* KHW in semisolid AB agar (0.3% wt/vol). In addition, no significant difference was observed in the swimming motility of *BPSS0805* complement. The KHW/pUCP28T-*BPSS0805* exhibits a small increase in swimming motility phenotype relative to the parental wild type *B. pseudomallei* KHW.

3.6.2 *cdpA* mutant exhibited an aflagellated and elongated phenotype

Absence of swimming motility in KHW*cdpA*::Tet might be due to aberrant flagellation or a flaw in the rotation frequency of the flagella. Analyses of the bacterial morphology using TEM revealed that the KHW*cdpA*::Tet mutant was aflagellated and 1.5 X more elongated than wild type KHW. A distinct flagellum was observed in the wild type bacteria but notably absent in the mutant. In addition, KHW*cdpA*::Tet was much elongated and approximately twice the length of wild type *B. pseudomallei* KHW (Fig. 15 A and B). These morphological changes were restored in the *cdpA* complemented mutant and KHW/pUCP28T-*cdpA*, which appeared similar in size to the wild type *B. pseudomallei* KHW (Fig. 15 C and D). Hence, a 40% reduction in intracellular c-di-GMP in the KHW/pUCP28T-*cdpA* strain did not affect flagellation and cell length. In addition, the introduction of full length *cdpA* restored flagella formation in the bacteria as observed in *cdpA* complemented mutant (Fig. 15 C), indicating a possible role for c-di-GMP in the regulation of bacteria flagella formation in *B. pseudomallei.*

3.6.3 *BPSS0805* null mutant did not alter the morphology of *B. pseudomallei* KHW

As *cdpA* null mutation led to a change in cell morphology and flagellar development in *B. pseudomallei*, likewise, transmission electron microscopy studies were performed to ascertain any morphological differences between KHW*BPSS0805*::Km and wild type KHW. Interestingly, the TEM studies showed that size of the KHW*BPSS0805*::Km mutant were similar to that of wild type *B. pseudomallei* and there is a notable change in the number of flagella per cell was observed in the mutant cells (Figure 16 A and B). In addition, the complemented

KHW*BPSS0805*::Km mutant and the KHW/pUCP28T-*BPSS0805* strain were morphologically similar to wild type *B. pseudomallei* KHW (Fig. 16).

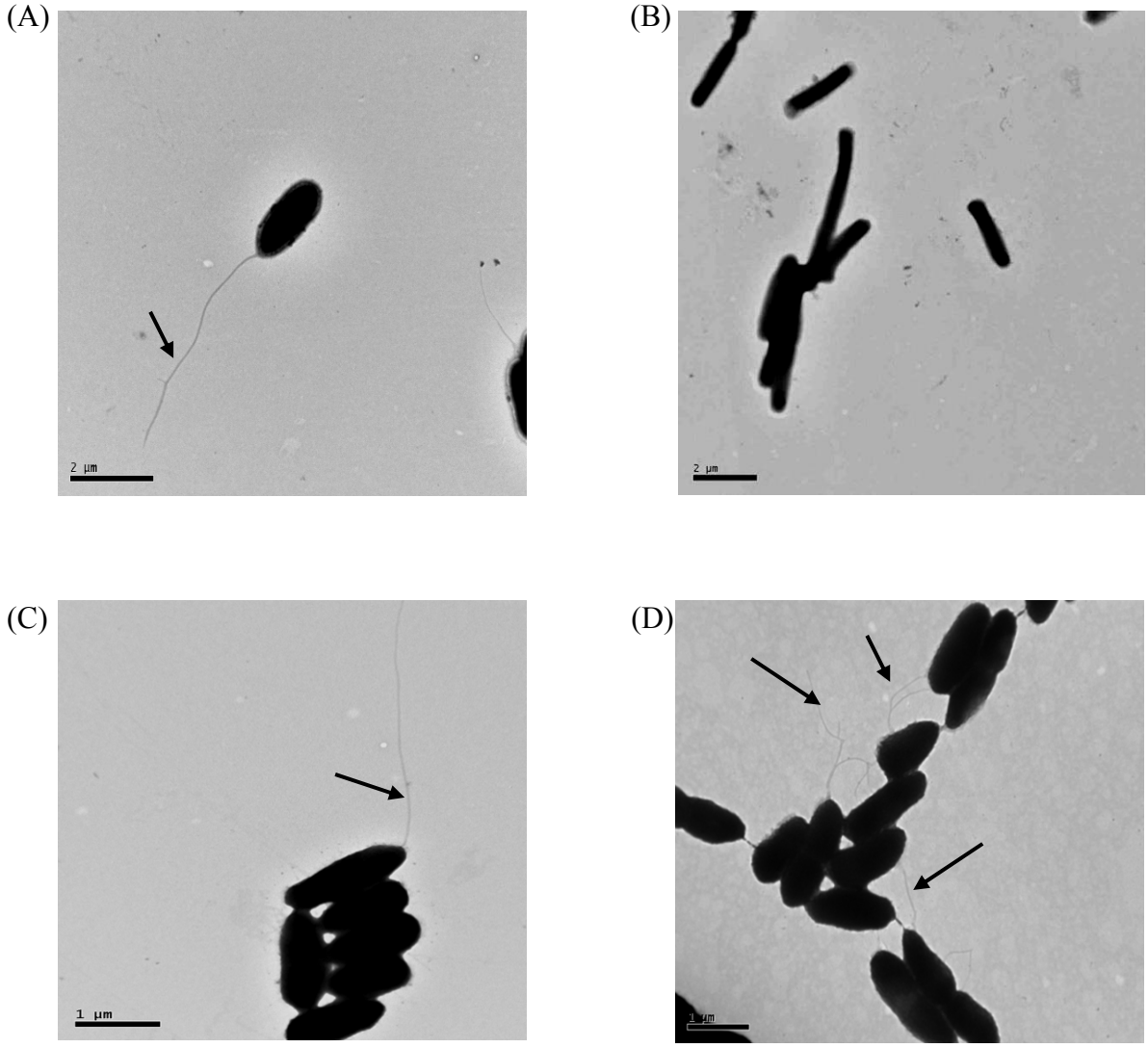


Fig. 15 Transmission electron micrographs showing *B. pseudomallei* KHW (A), KHW*cdpA*::Tet mutant (B), KHW*cdpA*::Tet/pUCP28T*cdpA* complemented mutant (C) and KHW pUCP28T*cdpA* (D). KHW*cdpA*::Tet mutant was aflagellated and significantly longer than wild type *B. pseudomallei* KHW. The sizes of *cdpA* complemented mutant and KHW/pUCP28T*cdpA* were restored back to wild type. In addition, flagella were clearly observed in the *cdpA* complemented mutant and KHW/pUCP28T*cdpA* though notably absent in *cdpA* mutant. Black arrows indicate flagella.

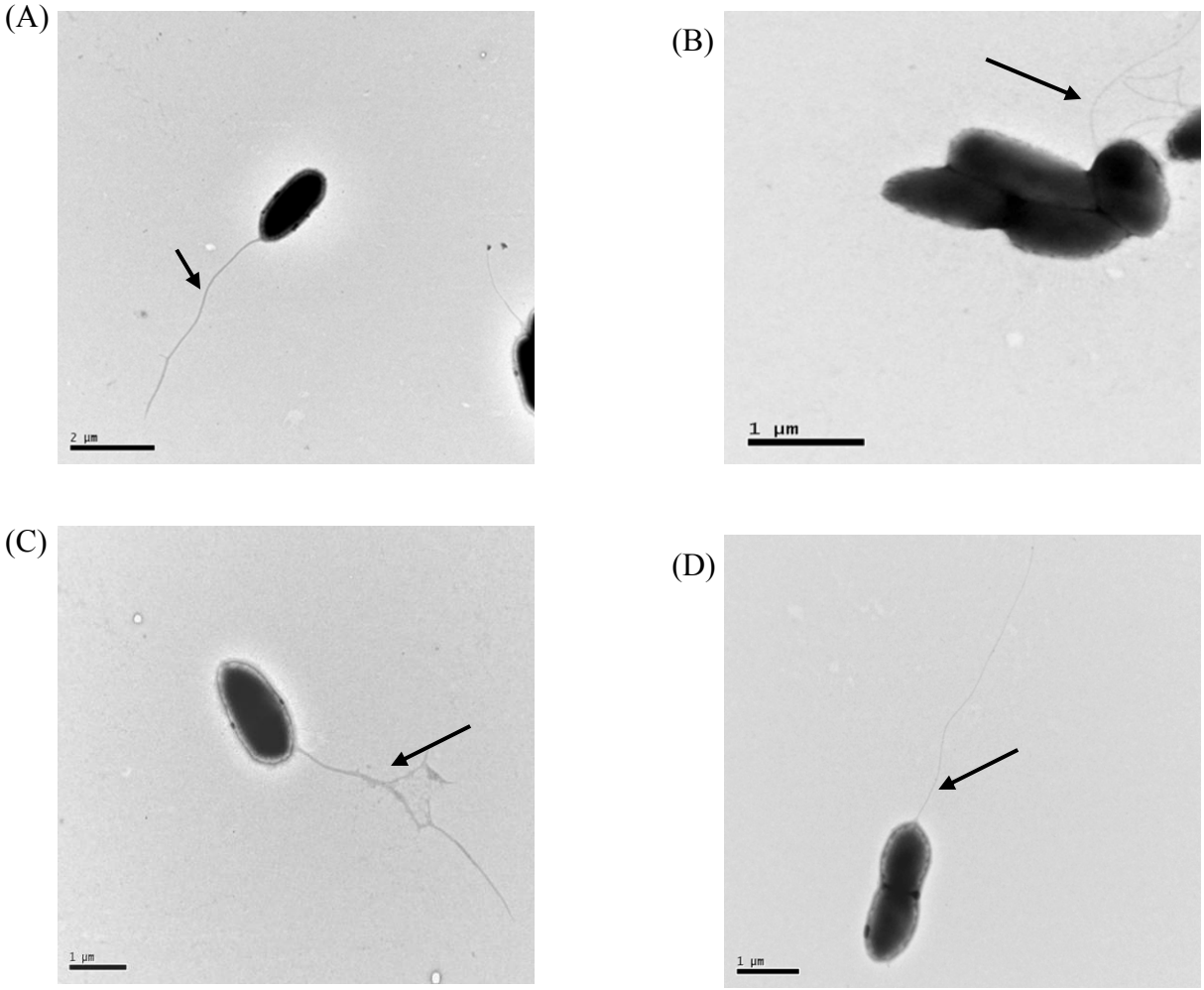


Fig. 16 Transmission electron micrographs showing *B. pseudomallei* KHW (A), KHW*BPSS0805*::Km mutant (B), KHW*BPSS0805*::Km/pUCP28T-*BPSS0805* complemented mutant (C) and KHW/pUCP28T-*BPSS0805* (D)

Visible flagella was observed in KHW*BPSS0805*::Km mutant wild type *B. pseudomallei* KHW, KHW*BPSS0805*::Km/pUCP28T-*BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805*. No obvious change in number of flagella for the different strains was noted. The sizes of the mutant cells were similar to wild type KHW. Black arrows indicate flagella.

3.6.4 CdpA regulates cellulose synthesis but BPSS0805 does not

The *BPSS1582* gene in the *B. pseudomallei* K96243 genome encodes a 766 amino acids cellulose synthase subunit B which shared 33.5% similarity in amino acids sequence with a *A. xylinus* cyclic di-GMP binding protein. Thus, the relationship between c-di-GMP levels and cellulose biosynthesis in *B. pseudomallei* was investigated by culturing the bacteria on LB agar plates without NaCl and supplemented with Congo Red dye (40 μg ml^{-1}) and Coomassie brilliant blue (20 μg ml^{-1}). The intensity of red coloration of the bacterial colonies and the level of cellulose biosynthesis were shown to be positively correlated with the level of cellulose biosynthesis (Simm *et. al.,* 2004). The KHW*cdpA*::Tet mutant formed reddish colonies while wild-type *B. pseudomallei* KHW formed pinkish white colonies, indicating a higher level of cellulose synthesis in the mutant (Table 8). The complemented KHW*cdpA*::Tet mutant showed reduced cellulose synthesis, similar to wild-type *B. pseudomallei* KHW.

The effect of the *BPSS0805* mutation on *B. pseudomallei* cellulose synthesis was similarly investigated, but the results showed no observable difference in the Congo red coloration of *BPSS0805* null mutant and the wild type *B. pseudomallei* KHW colonies, suggesting that BPSS0805 did not influence *B. pseudomallei* cellulose production. This observation is consistent with the findings that *BPSS0805* mutation did not alter the levels of intracellular c-di-GMP. Moreover, no difference was observed in the coloration of the colonies of *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805*, which further verifying that BPSS0805 have little effect on *B. pseudomallei* cellulose synthesis. (Table 9).

Table 8. Congo red binding assay for *B. pseudomallei* KHW, KHW*cdpA*::Tet mutant, *cdpA* complemented mutant and KHW/pUCP28T*cdpA*

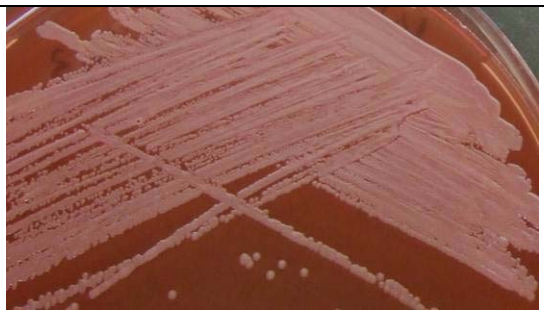
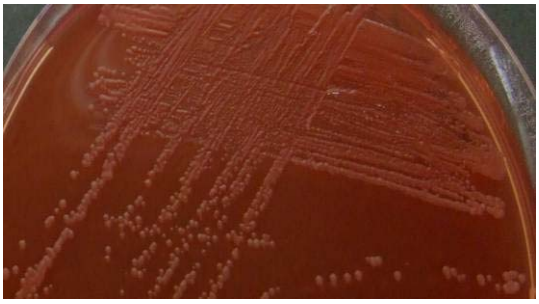
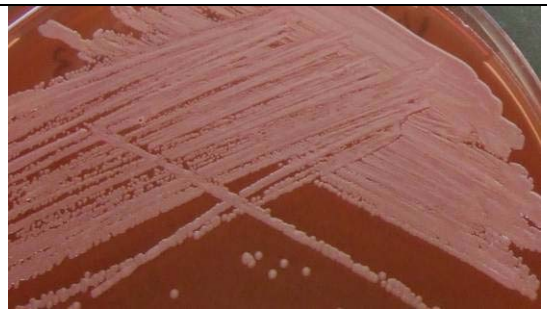
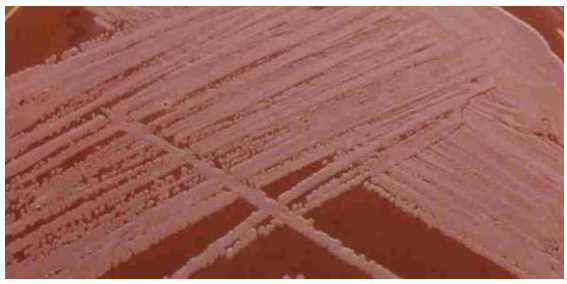
Bacterial Strain	Observations	
B. pseudomallei KHW (wild type)	Pinkish-white colonies observed	
KHW*cdpA*::Tet mutant	Reddish colonies observed	
KHW*cdpA* ::Tet/pUCP28T-*cdpA*	Pinkish-white colonies observed	
KHW/pUCP28T*cdpA*	Pinkish-white colonies observed	

Table 9. Congo red binding assay for *B. pseudomallei* KHW, KHW*BPSS0805*::Km mutant, *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805*

Bacterial Strain	Observations	
B. pseudomallei KHW (wild type)	Pinkish-white colonies observed	
KHW*BPSS0805*::Km mutant	Pinkish-white colonies observed	
KHW*BPSS0805* ::Km/pUCP28T-*BPSS0805*	Pinkish-white colonies observed	
KHW/pUCP28T-*BPSS0805*	Pinkish-white colonies observed	

3.6.5 CdpA inversely regulates bacterial cell aggregation but not BPSS0805

Based on previous studies conducted on *rpfG*, a c-di-GMP phosphodiesterase in *X. campestris,* a mutation in *rpfG,* led to an increase in bacterial cell aggregation, partly as a result of increase exopolysaccarides production (Dow *et. al.,* 2003). Thus, it is proposed that a mutation in *B. pseudomallei* c-di-GMP phosphodiesterase *cdpA* would similarly lead to an increase in bacterial cell aggregation. As expected, the results showed that when aggregation of KHW*cdpA*::Tet bacterial cells at the bottom of the tube was observed in 24 h-old static culture in LB medium (Fig. 17, tube 2). This phenomenon was not observed in wild-type *B. pseudomallei* KHW and the complemented KHW*cdpA*::Tet mutant (Fig. 17, tubes 1, 3 and 4).

Unlike *cdpA*, mutation in *BPSS0805* did not produce any changes in bacterial cell aggregation in static LB medium after 24 h as compared to wild type *B. pseudomallei* KHW (Fig. 17, Tube 1 and Tube 5). Consequentially, the *BPSS0805* complemented mutant and *B. pseudomallei* KHW/pUCP28T-*BPSS0805* also did not restore or reduce the level of bacteria cell aggregation in static LB culture, respectively (Fig. 17, Tube 6) and KHW (Fig. 17, Tube 7). Taken together, these results suggest that BPSS0805 may not be responsible for aggregation of *B. pseudomallei* cells in static culture.

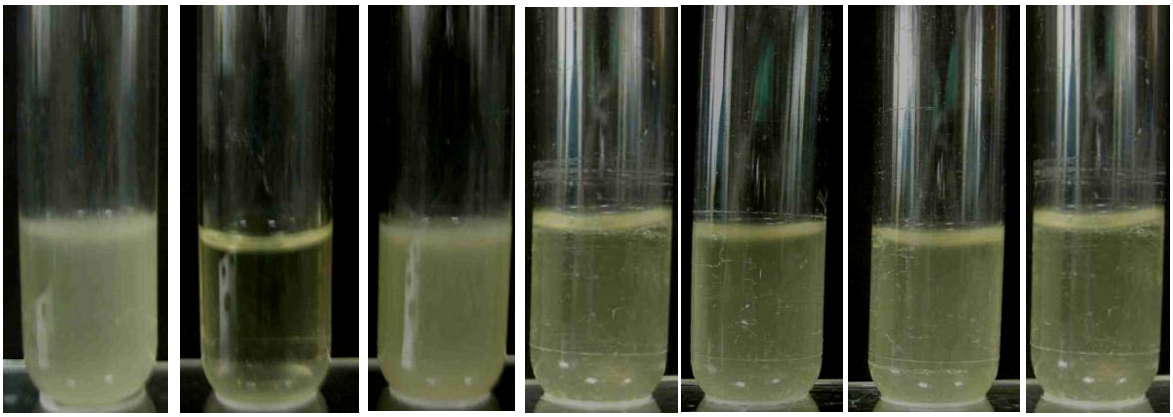


Fig. 17. Effects of *cdpA* and BPSS0805 mutation on the formation of cell aggregates by *B. pseudomallei.* Strains of KHW*cdpA*::Tet (Tube 2) grew in aggregated fashion, which settled at the bottom in static LB medium whereas wild type *B. pseudomallei* KHW (Tube 1), *cdpA* complemented mutant (Tube 3) and KHW/pUCP28T-*cdpA* (Tube 4) grew in a dispersed fashion. Also, *BPSS0805* mutant did not affect *B. pseudomallei* cell aggregation. No aggregation behavior was observed in *B. pseudomallei* KHW (Tube 1), *BPSS0805* mutant (Tube 5), *BPSS0805* complemented mutant (Tube 6) and KHW/pUCP28T-*BPSS0805* (Tube 7) after 24 h in static LB medium.

3.6.6 Effects of CdpA and BPSS0805 on biofilm formation

It is now well established that GGDEF-EAL proteins play an important role in the regulation of biofilm formation in wide range of bacteria as reviewed in Section 1.4. In a variety of bacteria such as *P. aeruginosa*, *S. typhimurium*, *Vibrio* spp., and *Y. pestis,* increased levels of c-diGMP were associated with enhanced biofilm formation while decreased intracellular levels of c-diGMP resulted in defective biofim initiation (Kulasakara *et. al.,* 2006; García *et. al.,* 2004; Hickman *et. al.,* 2005; Kirillina *et. al.,* 2004).

Hence, KHW*cdpA*::Tet mutant, which has a significantly higher level of intracellular c-di-GMP relative to wild type *B. psuedomallei*, was postulated to exhibit enhanced biofilm formation. Indeed, the KHW*cdpA*::Tet mutant produced more biofilm when compared to the wild-type *B. pseudomallei* (Fig. 18A). Biofilm formation in KHW*cdpA*::Tet mutant was increased ~3.7 times that in the wild type KHW. Moreover, it was noted that the complementation of KHW*cdpA*::Tet mutant with full length *cdpA* restored the level of biofilm formation back to the levels of wild type *B.pseudomallei*, thus validating the link between intracellular c-di-GMP levels and formation of biofilm.

Unlike the *cdpA* mutant, KHW*BPSS0805*::Km did not show any difference in biofilm formation when compared to the wild-type (Fig. 18B). This observation is expected as intracellular c-di-GMP levels in the *BPSS0805* mutant and wild-type *B. pseudomallei* KHW were similar. This results was statistically tested using ANOVA analysis with post-hoc Tukey's multiple comparison test and was found to have a P value of greater than 0.05 and thus, statistically insignificant.

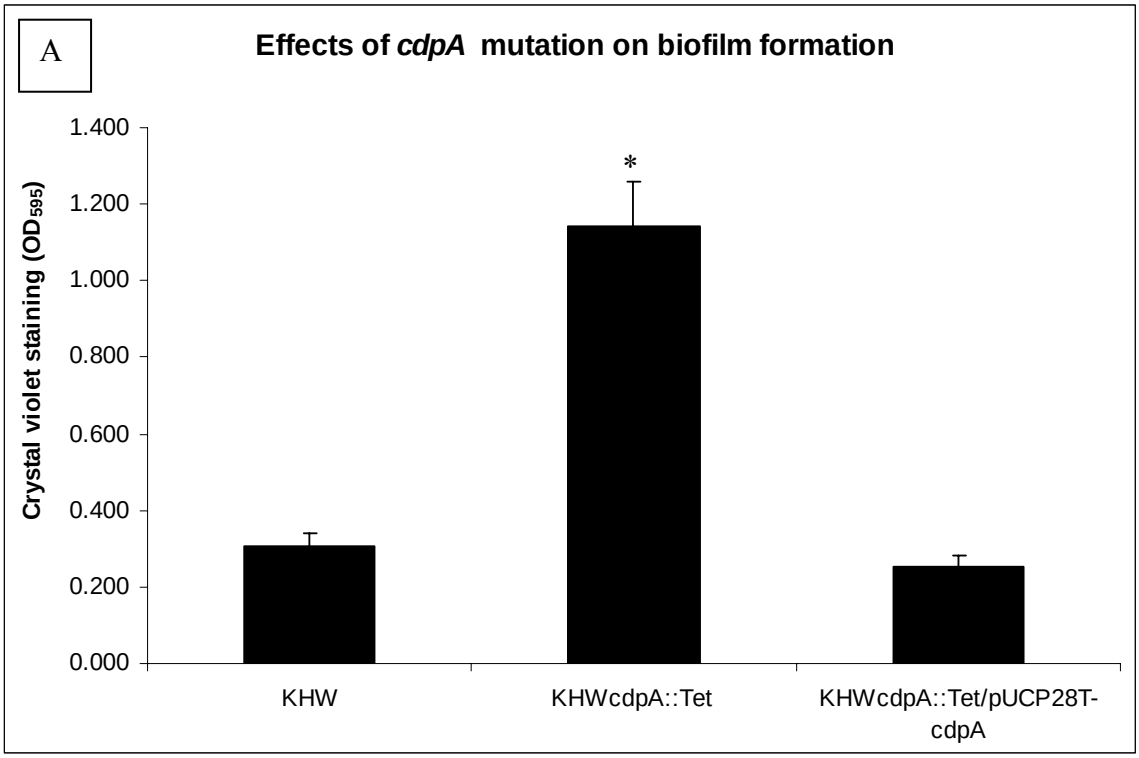


Fig. 18A. Effects of CdpA on *B. pseudomallei* biofilm formation
Quantitative representation of *B. pseudomallei* biofilm formation in 96 wells PVC microtitre plate. All strains were grown in AB medium supplemented with 0.2% glucose and 0.5% CAA. Biofilm formation was assayed after 20 h incubation at 30°C. KHW*cdpA*::Tet mutant showed enhanced biofilm formation of ~3.7 x times higher than parental *B. pseudomallei* KHW while its *trans* complementation restored the phenotype back to parental *B. pseudomallei* KHW level. An asterisk denotes statistically significant difference ($P < 0.05$) to the values of wild type *B. pseudomallei* KHW as judged by the ANOVA test.

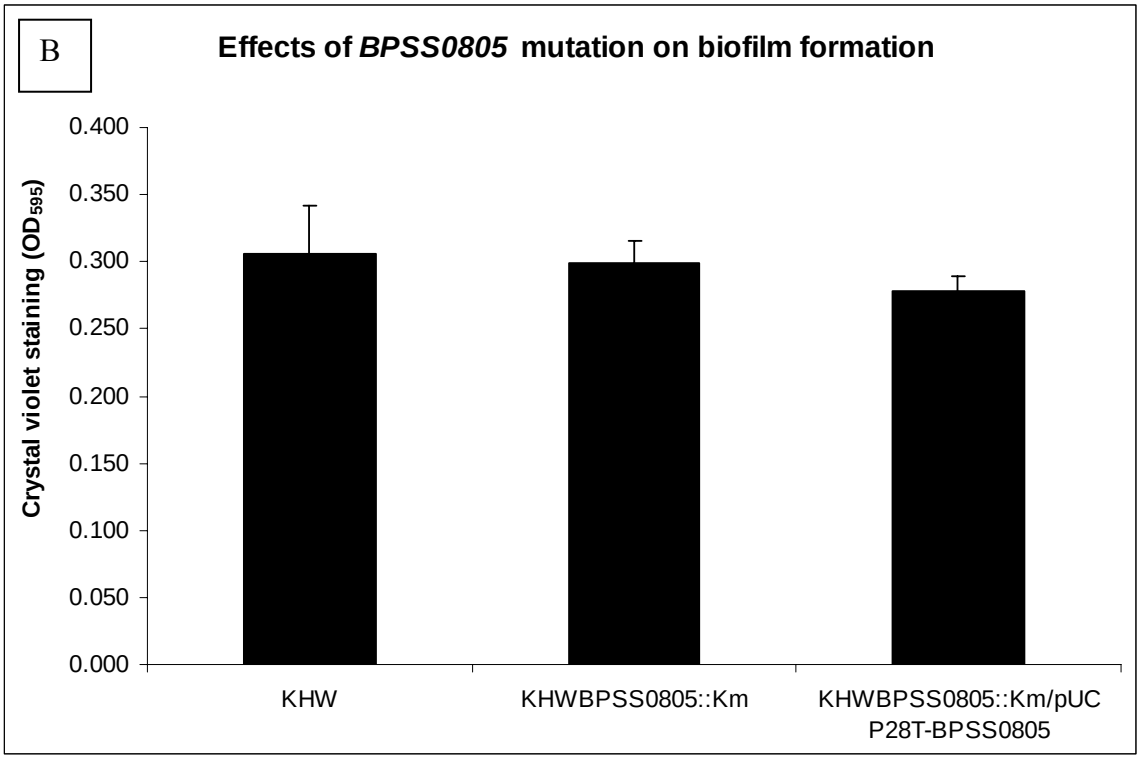


Fig. 18B. Effects of BPSS0805 on *B. pseudomallei* biofilm formation
Quantitative representation of *B. pseudomallei* biofilm formation in 96 wells PVC microtitre plate. All strains were grown in AB medium supplemented with 0.2% glucose and 0.5% CAA. Biofilm formation was assayed after 20 h incubation at 30°C. KHW*BPSS0805*::Km mutant and its complement stain, KHW*BPSS0805*::Km/pUCP28T-*BPSS0805* showed statistically similar level of biofilm formation relative to wild type *B. pseudomallei* KHW.

3.6.7 Absence of *cdpA* reduces mammalian cellular invasiveness by *B. pseudomallei*

The absence of flagella and impaired swimming motility of KHW*cdpA*::Tet mutant suggested that the *cdpA* mutant might exhibit reduced invasiveness of mammalian cells. To test this hypothesis, the ability of wild type *B. pseudomallei* and its isogenic mutants to invade human lung carcinoma cells (A549) were investigated. Bacterial invasion of A549 cells was attenuated by slightly more than threefold in KHW*cdpA*::Tet when compared to the wild type parental KHW (Fig. 19). Cell invasiveness was restored to wild-type levels in the complemented *cdpA* mutant, thus (Fig. 19). We conclude that the absence of CdpA reduces mammalian cell invasiveness by *B. pseudomallei.* In addition, cell invasiveness of KHW/pUCP28T-*cdpA* was similar to wild type *B. pseudomallei* KHW. Using ANOVA analysis with post-hoc Tukey's multiple comparison test, it was shown that no significant difference was found between KHW *B. pseudomallei* and KHW/pUCP28T-*cdpA* ($P > 0.05$).

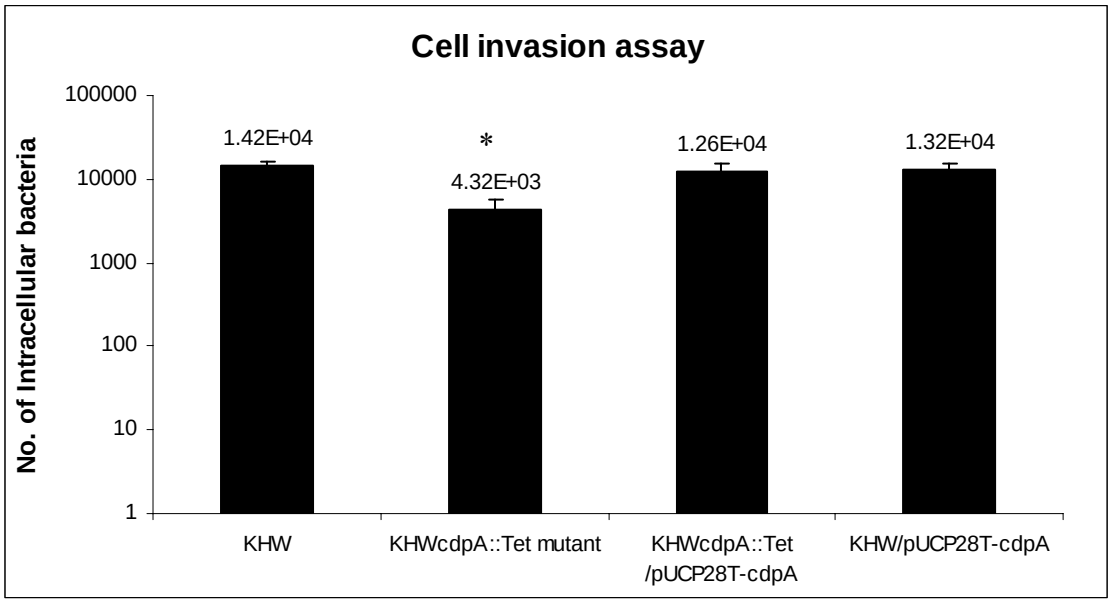


Fig. 19. Effects of *cdpA* on invasion of human lung carcinoma cells (A549). The number of intracellular bacteria cfu was threefold lower in the A549 cells exposed to 2 h incubation with KHW*cdpA*::Tet mutant compared to wild type *B. pseudomallei* KHW. Complementation of *cdpA* restored the phenotype, resulting in an almost equal number of intracellular bacteria cfu relative to wild type *B. pseudomallei* KHW. KHW/pUCP28T-*cdpA* also showed similar level of cell invasiveness compared to wild type KHW. Each bar represents the average of the triplicates from two independent experiments. An asterisk denotes statistically significant difference ($P < 0.05$) to the values of wild type *B. pseudomallei* KHW as judged by the ANOVA test.

3.6.8 CdpA is required for cell killing by *B. pseudomallei*

Apart from reducing cell invasiveness of *B. pseudomallei*, the *cdpA* mutation also reduced *B. pseudomallei* cytotoxicity on human macrophage cells (THP-1). Exposure of THP-1 cells to wild-type *B. pseudomallei* KHW for 4 h produced 37% killing of THP-1 cells, while exposure of THP-1 cells to the KHW*cdpA*::Tet mutant resulted in only 6% killing of the THP-1 cells (Fig. 20). Cytoxicity of the *cdpA* mutant was restored to wild-type after complementation with a functional copy of *cdpA*, thus confirming that the reduced cytotoxicity of the KHW*cdpA*::Tet mutant was due to the abolition of CdpA.

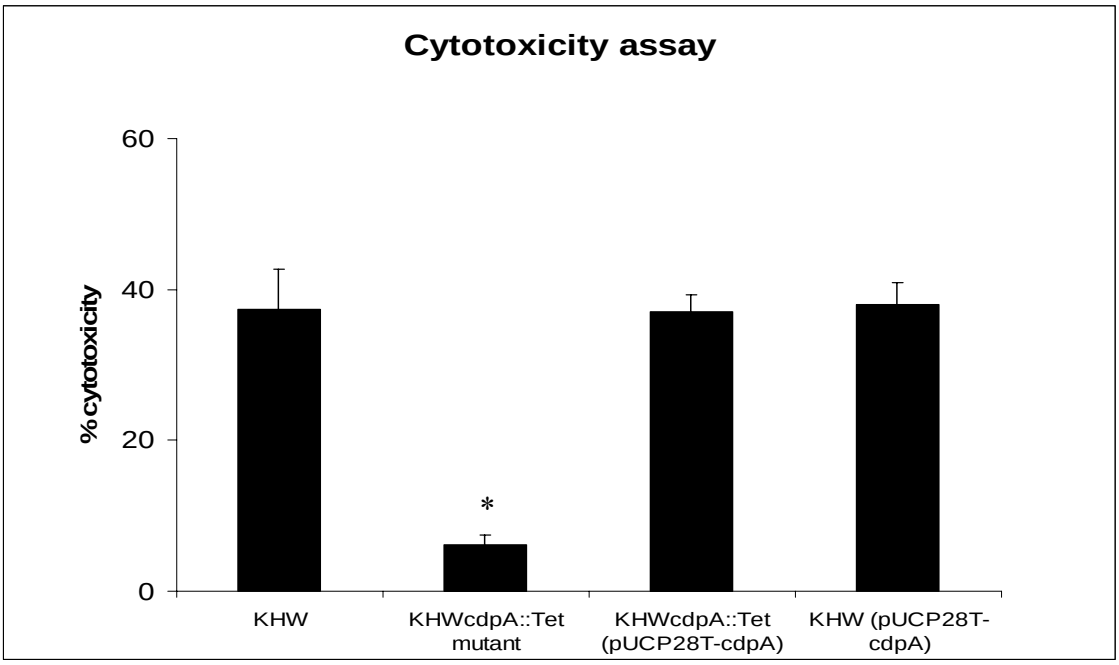


Fig. 20. Effects of *cdpA* mutation on cytotoxicity of *B. pseudomallei*.
The KHW*cdpA*::Tet mutant showed sixfold reduction in the killing of THP-1 cells after 4 h incubation compared to wild type *B. pseudomallei* KHW. Complementation of *cdpA* restored the phenotype. KHW/pUCP28T-*cdpA* also showed similar level of cytotoxicity compared to wild type *B. pseudomallei* KHW. Each bar represents the average of the triplicates from two independent experiments. An asterisk denotes statistically significant difference ($P < 0.05$) to the values of wild type *B. pseudomallei* KHW as judged by the ANOVA test.

3.6.9 BPSS0805 has minimal effects on *B. pseudomallei* mammalian cell invasiveness and cytotoxicity

Given the lack of significant differences in virulence associated phenotypes such as intracellular c-di-GMP levels, swimming motility and biofilm formation between the *BPSS0805* mutant and wild type *B. pseudomallei* KHW, it is hypothesized that the *BPSS0805* mutation is unlikely to affect the virulence of the pathogen. This was confirmed by the absence of any statistical difference in cell invasiveness of human lung carcinoma epithelial cell A549 by KHW*BPSS0805*::Km mutant relative to wild type *B. pseudomallei* KHW. Likewise, using ANOVA analysis with post-hoc Tukey's multiple comparison test, it was noted that there is no

statistical difference in cell invasiveness of *B. pseudomallei* KHW and KHW/pUCP28T-*BPSS0805* (Fig. 21).

The cytotoxicity assay was also carried out to determine any differences in the cytotoxicities between wild-type *B. pseudomallei* KHW and KHW*BPSS0805*::Km mutant. From the results, it was observed that both wild-type *B. pseudomallei* KHW and KHW*BPSS0805*::Km mutant resulted in ~37% killing of THP-1. Using ANOVA analysis with post-hoc Tukey's multiple comparison test, the differences in cytotoxicities between *B. pseudomallei* KHW and KHW*BPSS0805*::Km mutant was found to be statistically insignificant ($P>0.05$) (Fig. 22). The cytotoxicities of *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805* were also investigated using the same methodology. Exposure of THP-1 cells to *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805* for 4 h produced 36.7 % and 38.4% killing of THP -1 cells. Using ANOVA test, no statistical difference ($P>0.05$) was noted between *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805* when compared to wild type *B. pseudomallei* KHW (Fig. 22).

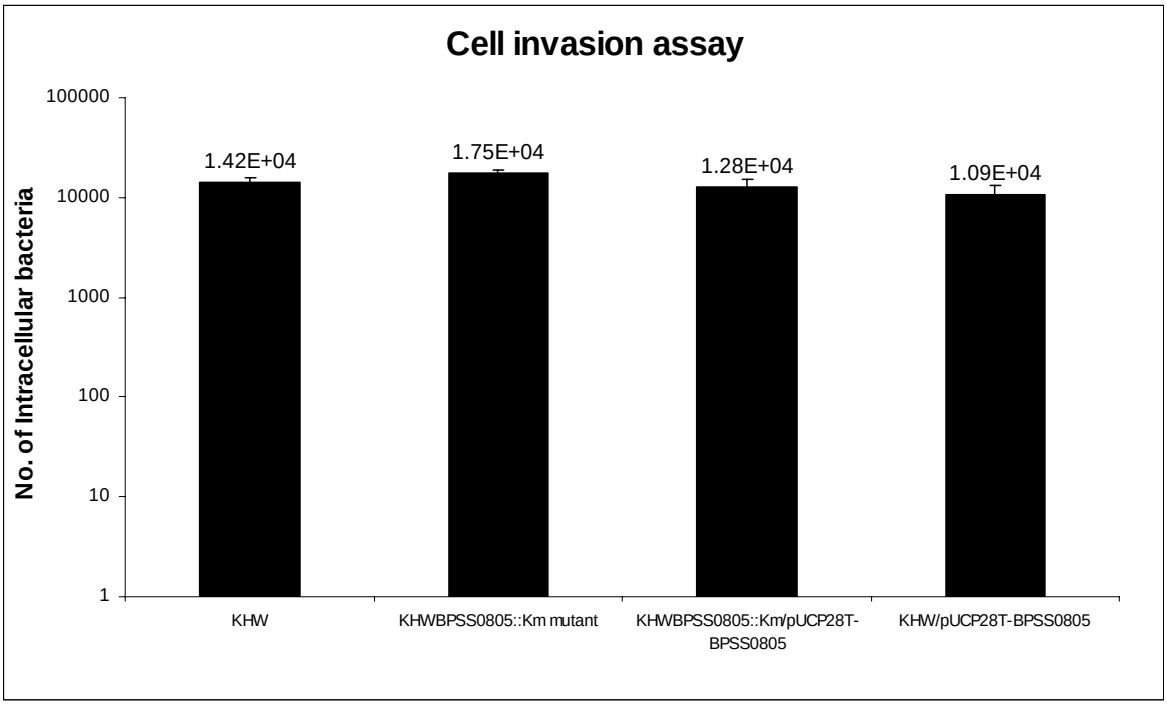


Fig. 21. *BPSS0805* mutation did not alter *B. pseudomallei* mammalian cell invasiveness

Invasion of A549 cells monolayer by *B. pseudomallei* and the KHWBPSS0805::Km mutant. No statistically significant increase between the intracellular bacteria cfu was noted after 2 h exposure to KHW*BPSS0805*::Km mutant. Similarly, no statistically difference in number of intracellular bacteria cfu was noted between *BPSS0805* complemented mutant, KHW/pUCP28T-*BPSS0805* and wild type *B. pseudomallei* KHW. Each bar represents the average of the triplicates from two independent experiments. Using ANOVA test, no statistical difference ($P>0.05$) was found between KHW*BPSS0805*::Km mutant, *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805* when compared to wild type *B. pseudomallei* KHW.

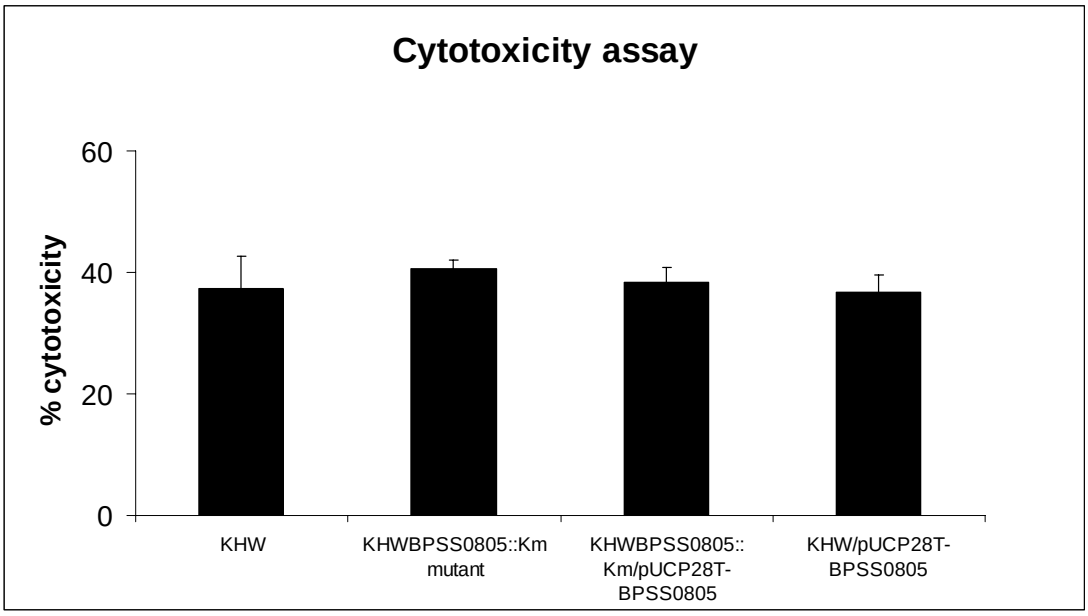


Fig. 22. Cytotoxicity of *B. pseudomallei* was not altered by *BPSS0805* mutation
The KHW*BPSS0805*::Km mutant did not show any significant difference in cytotoxicity of THP-1 compared to wild type *B. pseudomallei* KHW. Complementation of *BPSS0805* and KHW/pUCP28T-*BPSS0805* also showed similar level of cytotoxicity compared to wild type *B. pseudomallei* KHW. Each bar represents the average of the triplicates from two independent experiments. Using ANOVA test, no statistical difference (P>0.05) was found between KHW*BPSS0805*::Km mutant, *BPSS0805* complemented mutant and KHW/pUCP28T-*BPSS0805* when compared to wild type *B. pseudomallei* KHW

4.0 Discussion

4.1 *In* silico analysis of GGDEF-EAL proteins in *B. pseudomallei*

The bacterial second messenger c-di-GMP was recently demonstrated as a key player in bacterial signal transduction pathways, regulating cellular processes including cell surface properties, and in turn, motility, biofilm formation and virulence. To date, a number of studies on the regulation of bacteria phenotypes have been conducted on different bacteria species including *G. xylinus, E. coli, P. aeruginosa, S, typimurium, C. crescentus, V. chlorea, V. parahaemolyticus, V. fischeri, X. campestris* etc (Amikam and Benziman, 1989; Weber *et. al.,* 2006; Hickman *et. al.,* 2005; Kader *et. al.,* 2006; Aldridge *et. al.,* 2003; Ferreira *et. al.,* 2008; O'Shea *et. al.,* 2006; Lim *et. al.,* 2006; Ryan *et. al.,* 2007). In these bacteria, the intracellular level of c-di-GMP is controlled by proteins containing the GGDEF and EAL domains, which are responsible for the generation and hydrolysis of c-di-GMP respectively (Tal *et. al.,* 1998; Tamayo *et. al.*, 2005). Although the regulation of adaptive phenotypes by c-di-GMP levels had been studied in several bacteria species, to date, no similar documented study has been carried out in *B. pseudomallei.*

In this study, the GGDEF-EAL proteins of *B. pseudomallei* were identified based on the annotated sequences of *B. pseudomallei* K96243 genome. Out of a total of 16 putative GGDEF-EAL genes in *B. pseudomallei,* five encode only the GGDEF domain, six only the EAL domain and the remaining five encode both domains. Having multiple GGDEF-EAL protein in the genome is consistent with other bacteria species. *S. Typhimurium*, for instance, harbors five proteins with GGDEF, seven proteins with EAL domain and seven proteins with both domains (Kader *et. al.,* 2006). *E. coli* K-12 has 19 GGDEF and 17 EAL domain proteins, with an overlap of seven proteins containing both domains (Méndez-Ortiz *et. al.,* 2006). In *P. aeruginosa*

PAO1, 17 different proteins with a DGC domain, 5 with a PDE domain, and 16 that contain both of these domains were identified (Kulasakara *et. al.,* 2006) and lastly, in *V. cholerae,* there are 31 genes that encode GGDEF proteins, 12 EAL proteins and 10 encode proteins with both domains (Lim *et. al.,* 2006).

Apart from the 16 GGDEF-EAL proteins, *B. pseudomallei* also encodes two HY-GYD domain genes, *BPSL0704* and *BPSS1648,* located on chromosome 1 and 2, respectively. As shown by Ryan and his colleagues, the HY-GYD domain is responsible for the degradation of c-di-GMP, functioning as a phosphodiesterase in *X. campestris* (Ryan *et. al.,* 2006). Hence, it is possible that both these genes in *B. pseudomallei* might also function as phosphodiesterases to regulate the intracellular concentration of c-di-GMP level *in vivo*. Future work involving *in vitro* functional characterization of PDE activities of the recombinant proteins would be able to ascertain whether this is true.

This redundancy of paralogous proteins in a bacterium is a challenge for the functional studies that aim to elucidate the physiological role of c-di-GMP signaling pathways. The numerous GGDEF – EAL proteins encoded by the bacteria raised questions of how the bacterium is able to coordinate the expression and activity of these proteins to tightly control c-di-GMP and more importantly, how these differences affect the phenotypes regulated by this ubiquitous second messenger. Several hypotheses have been put forward to account for the large numbers of GGDEF-EAL proteins in bacteria. These include: a high level of functional redundancy in activities of these proteins (Kulesekara *et al.,* 2006), a complex, hierarchical network in which the activities of each GGDEF-EAL protein is highly differentiated (Kader *et. al.,* 2006) and, the possibility of temporal or spatial

regulatory mechanisms that allow for functional compartmentalization (Güvener and Harwood, 2007).

The activities of GGDEF-EAL proteins in bacteria may be regulated through the differential activation by environmental stimuli. The SMART analysis of the *B. pseudomallei* showed that five of the *B. pseudomallei* proteins are linked to a signal sensor domain, suggesting that environmental signals can be perceived and transmitted by c-di-GMP signaling network(s). Although we have not established what these signals are in *B. pseudomallei,* some of the environmental signals transmitted via c-di-GMP signaling pathways in other bacteria include: oxygen for *A. xylinum* (Chang *et. al.,* 2001), blue light for *E. coli* (Rajagopal *et. al.,* 2004), red/far red light for *R. sphaeroides* (Tarutina *et. al., 2006)* and nutrient starvation for *P. putida* (Gjermansen *et. al.,* 2006). As GGDEF-EAL domain proteins are commonly found in bacteria living in diverse environmental niches, it was not surprising that *B. pseudomallei*, an environmental saprophyte and facultative human and animal pathogen, should encode 16 different GGDEF-EAL proteins. It has been observed that organisms inhabiting stable niche environment tend to possess relatively simple signal transduction systems as compared to organisms that survive in harsh living conditions in diverse ecological niches, which demand a more complex signaling network to provide a more nimble response to changing environmental conditions (Galperin, 2005).

From these 16 different GGDEF-EAL proteins, two of them BPSL1263 and BPSS0805 were selected for further investigations. *In silico* analysis of BPSL1263 revealed that it is a composite protein, consisting of a 182 amino acids GGDEF domain and a 247 amino acids EAL domain arranged in tandem. These findings are in line with GGDEF-EAL domains from other bacteria, which have been shown to be

approximately 170 amino acids and approximately 250 amino acids in length respectively (Tal *et. al.,* 1998). The identities of the CdpA's GGDEF and EAL domains were further confirmed by their low E values of 1.1e-34 and 5.9e-115, respectively, against the Pfam database's GGDEF domain (Pfam Accession No. PF00990) and EAL domain (Pfam Accession No. PF00563).

Interestingly, it is noted that unlike the other GGDEF domain proteins in *B. pseudomallei,* there was no conserved GG(D/E)EF motif in the BPSL1263 protein. Instead this was replaced by the amino acids ASDKF. This conspicuous difference was critical as structural analysis of the DGC PleD from *C. crescentus* also showed that the second glycine in this conserved motif is essential for the catalytic function of the DGC (Chan *et. al.,* 2004). Similarly, *P. aeruginosa* PA2567, which contains ASNEF instead of GG(D/E)EF conserved motif, was shown to function as a c-di-GMP phosphodiesterase *in vitro* (Ryan *et. al.,* 2006).

Apart from the GGDEF-EAL domains, BPSL1263 also contained a transmembrane spanning region and a PAS domain at its N-terminal. This PAS domain is commonly found in bacteria signal transduction proteins, in particular, those associated with the sensing of oxygen and redox potential (Zhulin *et. al.,* 1997). For instance, *E. coli* Aer protein, which contains the highly conserved PAS domain, is responsible for the bacterial adaptive response to changes in the concentration of oxygen, redox carriers and carbon sources (Rebbapragada *et. al.,* 1997). In another example, the PAS domain of the *G. xylinus* PDE1, which bind to molecular oxygen, was found to inhibit its c-di-GMP phosphodiesterase activities (Chang *et. al.,* 2001). Taken together, these *in silico* predictions of BPSL1263 suggests that it is likely to be membrane-bound with a sensory domain.

In silico analysis of BPSS0805 as a GGDEF-EAL domain revealed the lengths of its GGDEF and EAL domains (173 and 248 amino acids, respectively), which resembled those found in other bacteria. The low E values of 1.5e-65 and 7.10e-98 for the GGDEF and EAL domains, respectively, strongly support the classification of BPSS0805 as a GGDEF-EAL protein. Unlike in BPSL1263, BPSS0805 contained the highly conserved GG(D/E)EF domain, suggesting that it probably functioned as a DGC. Pairwise alignment of BPSS0805 also revealed a high level of similarity with several previously investigated DGC proteins. However, no statistical significant difference in the intracellular c-di-GMP levels was detected in the BPSS0805 null mutant and wild type *B. pseudomallei* KHW, hence suggesting despite its high levels of homology with other DGC proteins, BPSS0805 might be a non-functional GGDEF-EAL protein, or the null mutation of *BPSS0805* actually triggered compensatory mechanisms in other GGDEF-EAL proteins to maintain the homeostatic level of c-di-GMP in *B. pseudomallei*.

Two MHYT domains (named after its conserved amino acid motif, methionine, histdine and tyrosine) present at the N-terminal (amino acids residues 52-113 and 115-177) are integral membrane sensor domains that are involved in the detection of oxygen, carbon monoxide and nitrogen oxide. The detection of these environmental signals by MHYT domains could possibly regulate the activities of the adjacent GGDEF-EAL domains (Galperin *et. al.*, 2001; Galperin, 2004). The prediction that eight transmembrane helices are present at the N terminal of BPSS0805 suggests that it is also likely to be bound to the bacterial membrane. Together, the presence of signal receiver MHYT domains, the transmembrane segments and the GGDEF-EAL domain suggests that BPSS0805 play a role in regulating the intracellular c-di-GMP levels.

4.2 BPSL1263 (CdpA) affects the intracellular c-di-GMP level of *B. pseudomallei*

Based on our *in silico* analysis, *BPSL1263* was shown to encode both GGDEF and EAL domains but notably, it lacks the conserved GG(D/E)EF motif, which was demonstrated to be essential for DGC activities in several bacteria (Chan *et. al.,* 2004; Ryan *et. al.,* 2006). As such, it is postulated that BPSL1263 would most likely function as a c-di-GMP phosphodiesterase. Hence, to elucidate the exact catalytic roles of BPSL1263, two main approaches were taken.

Firstly, based on the studies by Schmidt *et. al.* (2005) and Tamayo *et. al.* (2005), several attempts were carried out to overexpress BPSL1263 as a recombinant protein and analyze its catalytic properties in a purified system *in vitro*. Although *BPSL1263* was successfully cloned into expression vector pET-28a and overexpressed in *E. coli* Rosetta cells, the overexpressed recombinant BPSL1263 (rBPSL1263) formed inclusion bodies in the host *E. coli* cells. And despite expressing the protein under several conditions including different temperatures and concentrations of IPTG, no soluble rBPSL1263 was purified (data not shown). Subsequently, *BPSL1263* was cloned into pMAL-c2x vector which carries a solubility enhancing N-terminal maltose binding protein and overexpressed in *E. coli* DH5α (Fox and Waugh, 2002). Nevertheless, overexpressed rBPSL1263 still formed insoluble inclusion bodies and thus greatly hindered the purification of native BPSL1263, which is required for the downstream *in vitro* characterization of its catalytic functions (data not shown). Summing up, it is postulated that BPSL1263's properties as a membrane bound protein greatly affected its solubility and consequently, its native state purification.

Another approach, which involved the analysis of intracellular c-di-GMP levels in isogenic GGDEF-EAL knock out mutants, is based on the studies which

showed that mutation of the EAL-encoding gene *vieA* in *V. cholera* resulted in increased levels of c-di-GMP in nucleotide extracts (Tischler and Camilli, 2004). These analyses of the intracellular levels of nucleotides had also been documented in several bacteria including *G. xylinum* (Ross *et. al.,* 1991), *S. typimurium* (Simm *et. al.,* 2004) and *P. aeruginosa* (Kulasakara *et. al.,* 2006).

It is postulated that the difference in the levels of intracellular c-di-GMP in *BPSL1263* mutant relative to *B. pseudomallei* KHW would suggest its roles in c-di-GMP turnover. Hence, an investigation of the intracellular level of c-di-GMP in wild type *B. pseudomallei* KHW and its isogenic *BPSL1263* mutant based on the methodology described by Simm *et. al.* (2004) was carried out. Our results showed that while stationary phase *B. pseudomallei* KHW had around 5 pmol of c-di-GMP per mg of wet cell weight, the levels of c-di-GMP in *BPSL1263* knockout mutant was almost eight times higher at 40 pmol per mg of wet cell weight. This accumulation of c-di-GMP could either be due to the lack of EAL domain mediated phosphodiesterase activity in the mutant or an increased diguanylate cyclase activity encoded by another GGDEF protein in *B. pseudomallei* KHW.

To further verify the roles of BPSL1263 *in vivo*, the intracellular levels of c-di-GMP of the *BPSL1263* complemented mutant were investigated. The results showed that complementation of the *BPSL1263* reduced the amount of intracellular c-di-GMP, restoring it close to the wild type levels. This ability to complement the mutation by introducing the pUCP28T plasmid expressing *BPSL1263* confirmed that the changes in intracellular c-di-GMP levels were not due to a second-site or polar mutation that might occurred during its construction. From the results, it was also observed that the introduction of a full length *BPSL1263* into wild type *B. pseudomallei* KHW further decreased the amount of intracellular amount of c-di-

GMP, which is consistent with studies in *S. typimurium* and *Shewanella oneidensis*, whereby the overexpression of EAL domain protein *yhjH* resulted in a reduction in the amount of intracellular c-di-GMP (Simm *et. al.,* 2004; Thormann *et al.,* 2006). Taken together, these results suggested that the activity of BPSL1263 reduced the levels of intracellular c-di-GMP in *B. pseudomallei*.

4.3 Intracellular c-di-GMP levels was unaffected by the BPSS0805 null mutation

Although the *in silico* analysis of *BPSS0805* showed that both GGDEF and EAL domains were encoded by the gene, interestingly, *in vivo* analysis of the intracellular levels of c-di-GMP did not reveal much difference in the amount of c-di-GMP between the wild type *B. pseudomallei* and the isogenic *BPSS0805* mutant. These findings suggested that BPSS0805 might be a non-functional GGDEF-EAL protein, or the null mutation of *BPSS0805* actually triggered compensatory mechanisms in other GGDEF-EAL proteins to maintain the homeostatic level of c-di-GMP in the bacterium. It is also possible that c-di-GMP metabolic activities of BPSS0805 may require activating signals not present during the assays conducted in the study and to date, no studies has conclusively rule out the reciprocal inhibition of enzymatic activity by the DGC and PDE modules.

Functional redundancy among GGDEF-EAL proteins is actually a common theme in bacteria (Galperin, 2005). In *P. aeruginosa,* more than half of the predicted GGDEF domain proteins (10 out of 17) when ovexpressed in its parental strain did not result in any detectable change in intracellular c-di-GMP levels (Kulasakara *et. al.,* 2006). Such redundancy often makes the effects of knockout of individual GGDEF-EAL domain proteins subtle and thus not easily detected by the limits of sensitivity of the HPLC assay.

Though BPSS0805 does not have a direct effect in c-di-GMP turnover in *B. pseudomallei*, this protein could possibly serve a sensory and/or regulatory role *in vivo*. Its MHYT domains could play a role in the sensing of external environmental changes such as oxygen or carbon dioxide levels while its enzymatically inactive GGDEF-EAL domain could sequester c-di-GMP to regulate its *in vivo* concentrations.

These possible roles of BPSS0805 will not be easily elucidated by the *in vivo* functional analysis of *BPSS0805* mutant involving the isolation of total intracellular nucleotides as the methodology assumed that c-di-GMP is freely diffusible in the cells and hence, does not account for the possibility of localization of c-di-GMP in the bacterium. It is possible that the GGDEF-EAL proteins might be localized within the cell poles resulting in spatial localization of c-di-GMP. Hence, in such cases, even though the total amount of intracellular c-di-GMP isolated between the mutant and wild type bacteria is similar, the *in vivo* spatial distribution of c-di-GMP may vary considerably (Shapiro *et. al.,* 2002; Güvener and Harwood, 2007).

4.4 Phenotypes of the *cdpA* and *BPSS0805* null mutants

As reviewed in Section 1, it is now well established that the c-di-GMP concentration positively regulates bacterial phenotypes, including sessility, biofilm formation, expression of adhesive extracellular matrix components, but negatively influences bacterial phenotypes, such as motility and virulence. However, given the diverse nature of bacteria signaling network, it will be overly simplistic to assume that all bacterial phenotypes are regulated in similar fashion. Due to the presence of numerous paralogous GGDEF-EAL proteins in the bacterium, it is clear that not every GGDEF-EAL proteins will be involved in the regulation of its phenotypes. For instance, Lim *et. al.* (2006) showed that only four of the seven *V. cholera* GGDEF-

EAL proteins were known to regulate its colony rugosity and Garcia *et. al.* (2004) suggested that six out of eight GGDEF domain proteins in *S. typimurium* exhibited functional redundancy in cellulose biosynthesis. To add on to the complexity of c-di-GMP signaling, the effects of GGDEF-EAL protein homologues tend to be species-specific. For example, the absence of MorA was shown to inhibit the motility of *P. putida* but not in *P. aeruginosa* (Choy *et. al.,* 2004).

Recently, it was shown that the existence of cyclic di-GMP riboswitches enables the second messenger to control the transcription and translation of many genes and exerts its global effects. These mRNA domains sense the changes in the levels of c-di-GMP and exert transcriptional control over the expression of genes involving c-di-GMP regulated phenotypes, such as flagellum biosynthesis, rugosity and virulence (Sudarsan *et. al.,* 2008). Hence, given the highly complex c-di-GMP signaling pathways and its regulatory influence on diverse phenotypes, the effects of *cdpA* and *BPSS0805* mutations on motility, flagellar development, cell aggregation, cellulose synthesis, biofilm formation and virulence were investigated.

4.4.1 Effects of c-di-GMP signaling on *B. pseudomallei* swimming motility

Bacteria motility is often a well coordinated adaptive behavior in response to environmental changes. Signal transduction is an integral part of this behavior and GGDEF-EAL proteins have been shown to play an important role in its regulation. From the numerous studies reviewed in Section 1.4, it was shown that high levels of intracellular c-di-GMP levels were found to inhibit motility while conversely, low levels of c-di-GMP promoted motility. A similar correlation between c-di-GMP and motility was observed in *B. pseudomallei* in this study. The null *cdpA* mutation, which led to an increase in c-di-GMP levels, significantly inhibited *B. pseudomallei* swimming motility. Complementation of the *cdpA* mutation restored this phenotype,

thus verifying the role of CdpA in the regulation of this adaptive behavior. In addition, the introduction of full-length *cdpA* into wild type *B. pseudomallei* KHW, which lowered intracellular levels of c-di-GMP by 40%, exhibited slight increase in bacterial motility. These findings were consistent with the observation that *BPSS0805* mutant, which did not alter the intracellular levels of c-di-GMP levels and consequently did not affect *B. pseudomallei* swimming motility.

In bacteria, swimming motility is mostly a flagella mediated movement (Jarrell and McBride, 2008). *E. coli* sense changes in environment cues and alter the direction of rotation of their flagella in swimming motility (Kojima and Blair, 2001) In *B. pseudomallei,* the lack of flagella in a flagellin structural gene *fliC* knockout mutant resulted in nonmotile phenotype (Chua *et. al.,* 2003). In *P. putida*, MorA mutation enhanced motility through its regulation of the timing of flagellar development (Choy *et. al.,* 2004). Hence it is postulated that the swimming motility defect of *B. pseudomallei cdpA* mutant could be due to changes in flagella number or a flaw in the rotation frequency of the flagella.

TEM photographs of the nonmotile *cdpA* mutant revealed that the bacterium, unlike *B. pseudomallei* KHW, is aflagellated and elongated. *Trans* complementation of *cdpA* restored its morphology back to wild type *B. pseudomallei* and KHW/pUCP28T-*cdpA*, appeared similar in size to the wild type *B. pseudomallei* KHW. These findings confirmed our hypothesis that the swimming motility defect of *cdpA* mutant is due to the changes in its flagella. This correlation between high levels of c-di-GMP and downregulation of flagella motility is not entirely new and was also observed in *S. typhimurium*, *V. cholerae, P. putida, P. aeruginosa*. In *S. typhimurium and V. cholerae,* overexpression of DGCs, AdrA and VCA0956 respectively, increased the intracellular c-di-GMP levels and significantly inhibited flagella

mediated swimming motility (Simm *et. al.,* 2004; Beyhan *et. al.,* 2006). However, further investigations are necessary to prove whether c-di-GMP regulation of flagella development is at the transcriptional, translational or post-translational level. Real time PCR analysis to investigate the expression levels of *fliC* transcript or comparative western blot analysis using anti FliC protein to investigate the amount of FliC protein could provide a better understanding of this regulatory pathway.

Interestingly, high c-di-GMP levels in *E. coli* overexpressing YddV, a PDE was found to lead to an elongation in shape of the bacterium. Genome-wide transcriptional profile showed that the transcription of 27 genes encoding membrane-associated proteins was dramatically decreased and notably, genes involved in cell division such as *ftsT* and *ftsX* were altered under this condition (Méndez-Ortiz *et. al.,* 2006). This suggested that high levels of intracellular c-d-GMP in the *cdpA* mutant might have similarly altered the transcription of membrane associated and cell division proteins, thus leading to its elongated cell morphology.

4.4.2 Effects of c-di-GMP signaling on *B. pseudomallei* cellulose production

The production of extracellular exopolysaccarides is a key step in the biofilm formation model proposed by Stooley *et. al.* (2002) (discussed in Section 1.6). EPS is required for the firm "irreversible" attachment of bacteria to the surfaces and cellulose was recently identified as an important matrix component in *Pseudomonas spp* biofilms (Ude *et. al.,* 2006).

The very first reports of the action of c-di-GMP *in vivo* were its association with bacteria cellulose synthesis (Ross *et. al.,* 1990). In *A. xylinus* and *A. tumefaciens*, c-di-GMP functions as an allosteric activator of cellulose synthase, whereby an increase in intracellular c-di-GMP directly led to an increase in cellulose synthesis

(Ross *et. al.,* 1997; Tal *et. al*., 1998). Furthermore, Simm *et. al.* (2004) showed that in *S. typimurium*, overexpression of the GGDEF domain protein AdrA led to elevated c-di-GMP levels, which activated cellulose biosynthesis while overexpression of the EAL domain protein YhjH reduced c-di-GMP levels and in turn, abolish cellulose biosynthesis. The constitutively active DGC WspR19 mutant in the *P. fluorescens* SBW25 wrinkly spreader phenotype showed elevated c-di-GMP levels and induced cellulose expression and biofilm formation (Malone *et. al*., 2007). This positive correlation between c-di-GMP and cellulose production was again noted in several other bacteria including *E. coli* and *V. cholera* (Weber *et. al.,* 2006; Lim *et. al.,* 2006). The qualitative methodology used for assaying cellulose production in bacteria involved growing them on Congo red agar plates. The Congo red dye has a strong, though apparently non-covalent affinity to cellulose fibres, thus providing a convenient assay for cellulose biosynthesis (Simm *et. al*., 2004). Changes in the outer membrane and surface properties of the bacteria, such as the presence of adhesive structures and exopolysaccarides, would produce a red coloration in this assay.

In *B. pseudomallei*, a cellulose synthase subunit B, BPSS1582 which shared 33.47% amino acids similarity with the c-di-GMP binding cellulose synthase subunit of *A. xylinus* was identified. Hence, it was hypothesized that the higher amount of c-di-GMP in *cdpA* mutant will allosterically activate *B. pseudomallei* cellulose synthase complex and result in increase cellulose production. From the results, *cdpA* mutant, which had higher levels of intracellular levels c-di-GMP, formed a higher intensity of red coloration of its colonies compared to wild-type *B. pseudomallei* KHW. In addition, complementation of *cdpA* restored the levels of cellulose synthesis back to the wild-type *B. pseudomallei* KHW. Taken together, these observations were

consistent with the hypothesis that c-di-GMP positively regulate cellulose synthesis in *B. pseudomallei.*

Furthermore, there was no observed difference in red coloration and therefore cellulose production between the colonies of KHW *B. pseudomallei,* BPSS0805 mutant, its *trans* complement strain and KHW*BPSS0805*::Km bacteria. As earlier findings showed that *BPSS0805* mutation did not alter c-di-GMP levels in the cells, these results were consistent with the findings that c-di-GMP is an important regulator of cellulose production in bacteria.

4.4.3 Effects of c-di-GMP signaling on *B. pseudomallei* bacteria aggregation

Multicellularity was once thought to be exclusive trait of eukaryotes while bacteria generally exist as free swimming unicellular organisms. However, recent studies, especially findings on the quorum sensing intercellular communication systems, have altered this opinion. Clumping of bacteria in the natural environment (aggregation) is now widely observed and known to bring about adaptive benefit,s including access to resources and niches, collective defense against microbes antagonists and adaptive mutation (Shapiro, 1998). For instance, aggregation of the plague bacterium *Yersinia pestis* can block food intake of both nematode worms and fleas, which increased its transmission by the flea vector (Hinnebusch *et. al.,* 1996). Bacterial aggregation in *B. pseudomallei* resulted in the formation of microcolonies, which was shown to greatly increase its interaction with eukaryotic cells and enhanced its colonization of host cells (Boddey *et. al.,* 2006).

Other than colonization of host cells, bacteria aggregation might also play a role in bacterial biofilm formation. In the biofilm model proposed by Stooley *et. al.* (2002), one of the mechanisms involved in the initiation of biofilm was the

aggregation/recruitment of cells from the bulk fluid to the developing biofilm. However, a study by Tolker-Nielsen and his colleagues using fluorescent labeled bacteria showed that microcolonies formed by aggregation of bacteria did not play a significant role in *P. putida* OUS82 or *Pseudomonas* sp. strain B13 biofilms. In fact, instead of bacteria aggregation from its environment, these microcolonies appeared to be formed from clonal growth of single cells attached to the substratum (Tolker-Nielsen *et. al.,* 2000).

In this study, KHW*cdpA*::Tet was formed cell aggregates at the bottom of the tube after growth in static culture in LB medium for 24 h but not wild type *B. pseudomallei* KHW, *cdpA* complemented mutant and KHW/pUCP28T-*cdpA*. This increase in bacteria aggregation and sedimentation of *cdpA* mutant could possibly be due to an increase in cellulose / EPS production, the lack of flagella mediated swimming motility or the compounding effects of both phenotypes. Such observations were similarly noted in studies of PDEs in other bacteria. To illustrate further, Dow *et. al.* showed that a mutation in *X. campestris* c-di-GMP PDE *rpfG,* resulted in increased intracellular c-di-GMP, reduced motility and consequently, an increase of bacterial cell aggregation (Dow *et. al.,* 2003). This positive correlation between increase c-di-GMP and bacteria aggregation was also observed in a mutant of the *E. coli* c-di-GMP phosphodiesterase, YciR (Weber *et. al.,* 2006).

In contrast, no changes in bacterial cell aggregation were observed in KHW*BPSS0805*::Km in static LB medium after 24 h as compared to the wild type. There was also no observable differences in bacterial aggregation patterns in *B. pseudomallei* KHW overexpressing *BPSS0805* cultured in static LB medium for 24 h. These results confirmed that elevated intracellular c-di-GMP levels are correlated to increase in aggregation of *B. pseudomallei* cells. It is likely that the sensory domains

of CdpA sense environmental signals and effect changes in gene expression by altering the intracellular c-di-GMP levels. Bacterial aggregation would be a consequence of such a signaling mechanism to confer adaptive benefits to the bacteria, including access to resources and niches, collective defense against antimicrobial agents, adaptive mutation and increasing colonization of host cells.

4.4.4 Effects of c-di-GMP signaling on *B. pseudomallei* biofilm formation

The majority of bacteria in most natural and pathogenic ecosystems are found in biofilm (O'Toole *et al*., 2000). Hence, its formation is an integral part of bacteria survivability in its natural environment. The development of biofilm is a multistep and complex process, whereby c-di-GMP regulated phenotypes, such as bacterial motility, aggregation and cellulose and EPS production were shown to be highly essential.

In a variety of bacteria such as *P. aeruginosa, Y. pesti*, *V. cholera,* intracellular c-di-GMP was generally found to activate biofilm formation. A comprehensive study of *P. aeruginosa* putative GGDEF-EAL genes by Kulasakara and his co workers, for instance, showed that overexpression of genes encoding DGC such as *PA5487* resulted in increased intracellular c-di-GMP levels and greatly enhanced biofilm formation (Kulasakara *et. al.,* 2006). In a separate study, mutation of *P. aeruginosa* c-di-GMP PDE encoding gene *PA5017* resulted in a mutant that displayed an increase in biofilm formation (Li *et. al.,* 2007). Similarly, in *Y. pestis,* the effects on biofilm formation were largely attributed to the putative DGC and PDE activities of HmsT and HmsP respectively. In fact, point mutation of HmsT GGDEF domain decreases its intracellular c-di-GMP levels and reduced its biofilm formation. Likewise, the

mutation in the EAL motif of HmsP led to an increase in c-di-GMP levels and significantly increased its biofilm formation (Kirillina *et. al.,* 2004).

Similarly, results shown in Section 3.6.6 concur with the data from these studies. High levels of intracellular c-di-GMP found in *cdpA* mutant were associated with increased biofilm formation in the mutant and complementation of the *cdpA* mutant using full-length *cdpA* restored the biofilm formation back to wild type *B. pseudomallei* level. In contrast, no significant difference in biofilm formation was observed in the *BPSS0805* mutant and wild type *B. pseudomallei* KHW suggesting that BPSS0805 did not have a functional role, either directly or indirectly through the regulation of c-di-GMP concentrations, in the biofilm formation of *B. pseudomallei.* These results were further verified by the lack of observable difference in biofilm formation between *BPSS0805* complement, KHW/pUCP28T-*BPSS0805* and wild type *B. pseudomallei* KHW.

Despite the strong correlation between intracellular c-di-GMP levels and biofilm formation, it might be overly simplistic to assume a simple "cause and effect" relationship between c-di-GMP levels and biofilm formation in bacteria. A multistep and critical process such as biofilm formation in bacteria is usually tightly regulated and involved multiple environmental signals. It is expected that the process will be involve cross talk and interplay between different proteins in the signaling network, including the quorum sensing systems. For instance, in *P. aeruginosa,* it was shown that *las* quorum sensing system is directly involved in the regulation of its biofilm formation and in *B. pseudomallei,* a functional *bps* quorum sensing system is required for the optimal biofilm development (Singh *et. al.,* 2000; Song *et. al.,* 2005). Thus, for future such studies on biofilm formation and development, it would be useful to adopt a global approach rather than to study each signaling network in isolation.

4.4.5 Effects of c-di-GMP signaling on *B. pseudomallei* virulence

The regulation of *B. pseudomallei* virulence associated and pathogenic processes, such as cell invasion; the ability to survive intracellularly; production of virulence factors, such as siderophore, protease etc, and cytotoxicity are often tightly controlled and occurred in response to environmental signals. In *B. pseudomallei,* the ability to invade mammalian cells is an important initial step in its pathogenesis. It was shown by Inglis and his team that flagellum mediated adhesion is a critical step in early stages of cellular invasion of eukaryotic cells *Acanthamoeba astronyxis* (Inglis *et. al.,* 2003). In *B. cepacia,* flagellum-mediated motility is essential for the bacteria invasion of A549 cells during both the initial establishment of contact between the bacteria and the host cells as well as its entry once contact has been established (Tomich *et. al*., 2003). In contrast, in a study conducted in our laboratory, Chua *et. al.* (2003) showed that flagella and motility appeared not to be necessary for *B. pseudomallei* to invade A549 human lung cells. No difference was actually noted in the cell invasiveness of an aflagellated mutant, KHW*fliC*ΔKm mutant and wild type *B. pseudomallei* KHW.

From the data obtained in this project, it was noted that the *cdpA* mutant showed a significant decrease in cell invasiveness compared to wild type *B. pseudomallei* KHW. Restoration to wild-type levels by complementation of the *cdpA* mutant verified that loss of cell invasiveness in the *cdpA* mutant was indeed due to the mutation of the *cdpA* and not due to a secondary site mutation. Separately, no significant differences in cell invasiveness between wild type *B. pseudomallei* KHW, *BPSS0805* mutant. At first glance, these findings, together with the observations that *cdpA* mutant is nonmotile and lacks flagella, seem to suggest that *cdpA* mutant's defect in invading eukaryotic cells is due to its immobility and lack of flagella.

However, given the understanding that lack of flagella in KHWΔ*fliC*Km mutant did not result in impaired cell invasiveness of *B. pseudomallei*, it is believed that directly assigning the defect in cell invasiveness of *cdpA* mutant to its immotibility and lack of flagella might be overly simplistic.

Besides cell invasiveness, the ability of *B. pseudomallei* to induce cell death in eukaryotic cells (cytotoxicity) is another key component of its virulence. From our data, it was shown that *cdpA* mutant exhibited reduced cytotoxicity compared to wild type *B. pseudomallei* KHW and the complemented *cdpA* mutant, thus providing preliminary evidence that high intracellular level of c-di-GMP could attenuate *B. pseudomallei* virulence. On the other hand, absence of any statistical difference between the cytotoxicity of the *BPSS0805* mutant and wild type *B. pseudomallei* KHW suggested that there is no functional role for BPSS0805 in the regulation of *B. pseudomallei* cytotoxicity. Furthermore, the introduction of the full length *BPSS0805* into *BPSS0805* mutant and wild type *B. pseudomallei* KHW did not result in any statistical differences in cytotoxicities compared to the wild type *B. pseudomallei,* thus further verified the lack of a functional role for BPSS0805 in the regulation of this phenotype.

However, despite these studies on the effects of c-di-GMP and bacterial virulence, the exact mechanisms and component of this regulatory network is still obscure. Hence, specific experiments such as comparative expression studies of virulence factors using Real Time-PCR between *B. pseudomallei* KHW, *cdpA* mutant and its *trans* complemented strain can be carried out to probe deeper into this complex pathway. Furthermore, comparative western blot analysis of cytotoxic proteins such as haemolysin, lipases and proteases can be carried out to determine the components involved in c-di-GMP regulated cytotoxicity.

Given the complexity of c-d-GMP signaling, further investigations are necessary to determine the precise roles, if any, of BPSS0805. A double knockout *cdpA* and *BPSS0805* mutant would allow us to determine whether these proteins shared overlapping functions, which were otherwise masked by the effects of either protein. Furthermore, it is also possible that the phenotypic assays conducted in this study are neither complex nor precise enough to investigate temporal influence of BPSS0805. Hence, a promoter$_{BPSS0805}$-*lacZ* fusion reporter plasmid can be constructed and utilized to study its expression under diverse environmental conditions. And with this knowledge, the phenotypic assays can then be improvised to elucidate the functions of BPSS0805.

5 Conclusion

CdpA and BPSS0805 are two *B. pseudomallei* GGDEF-EAL proteins. These two proteins were part of the 16 GGDEF-EAL proteins that were identified in this pathogen. Using mutagenesis and complementation studies, the functional role of CdpA as a phosphodiesterase in the metabolism of intracellular second messenger c-di-GMP was determined.

CdpA, through its regulation of intracellular levels of c-di-GMP was also showed to regulate biofilm formation and virulence of *B. pseudomallei,* either directly or through its influence on various bacterial phenotypes including swimming motility, cell length and flagella development, cell aggregation and sedimentation, cellulose biosynthesis. The *cdpA* mutant was also significantly attenuated cell invasion and cytotoxicity, thus providing preliminary evidence that high intracellular level of c-di-GMP could inhibit *B. pseudomallei* virulence.

On the other hand, GGDEF-EAL protein BPSS0805 had little effect on the intracellular levels of c-di-GMP. Consequently, the BPSS0805 null mutation did not

affect on phenotypes such as motility, cell aggregation, cellulose biosynthesis, biofilm formation. No significant difference was noted in mammalian cell invasion and cytotoxicity was noted between BPSS0805 mutant and wild type *B. pseudomallei.* Taken together, these results suggested functional redundancy of BPSS0805 *in vivo.*

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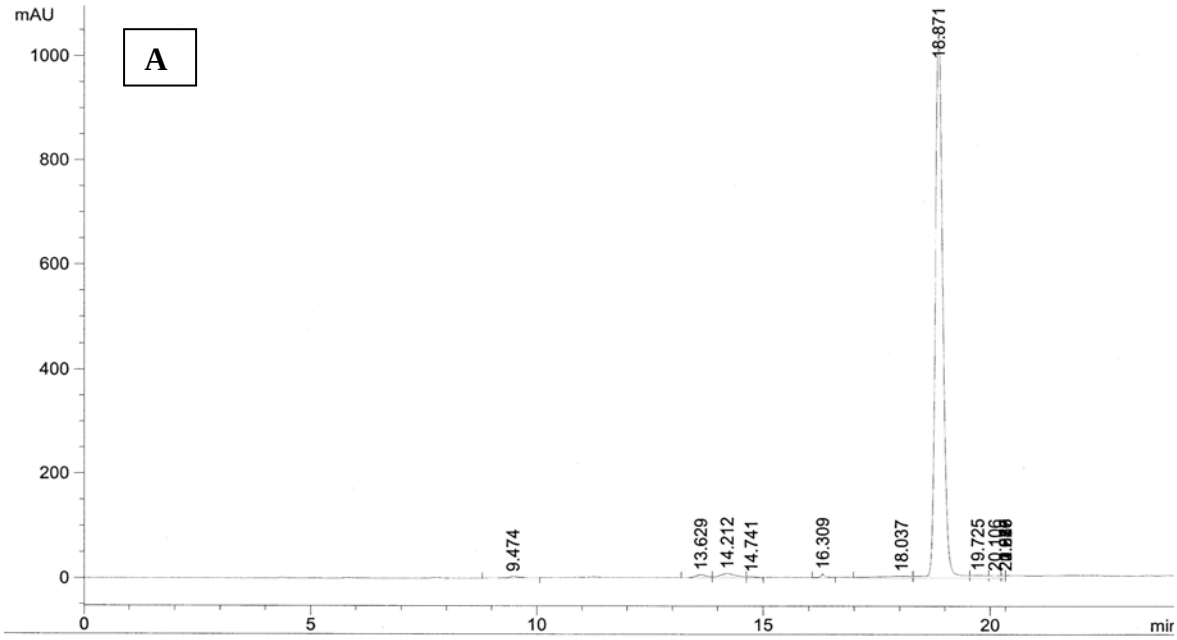
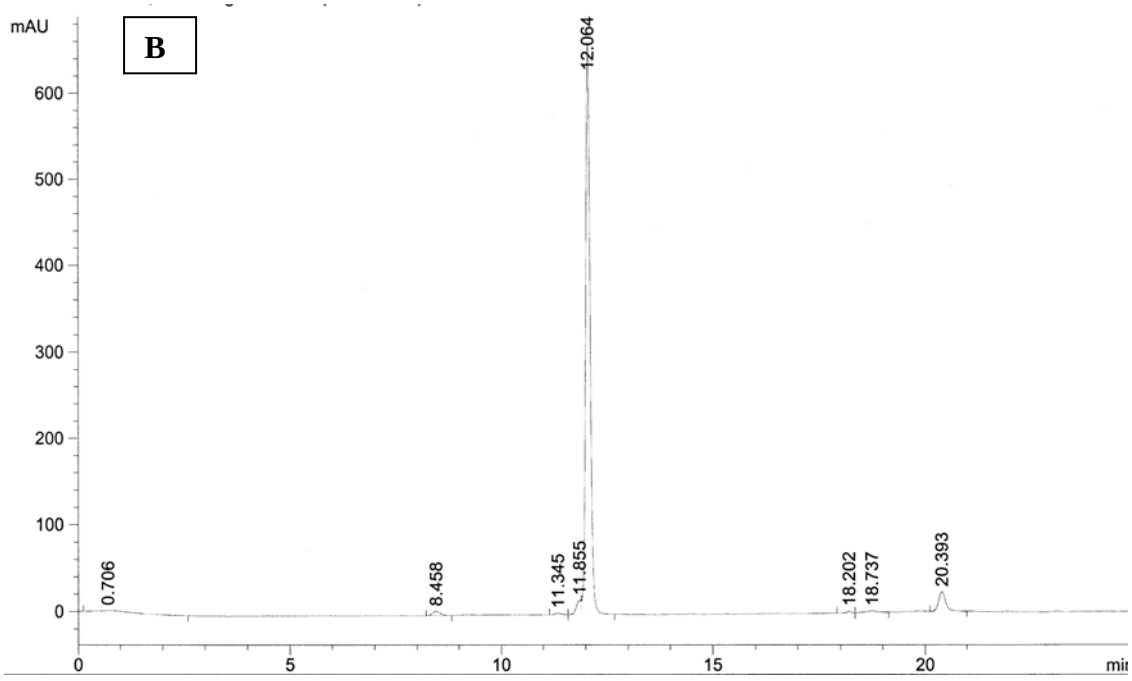
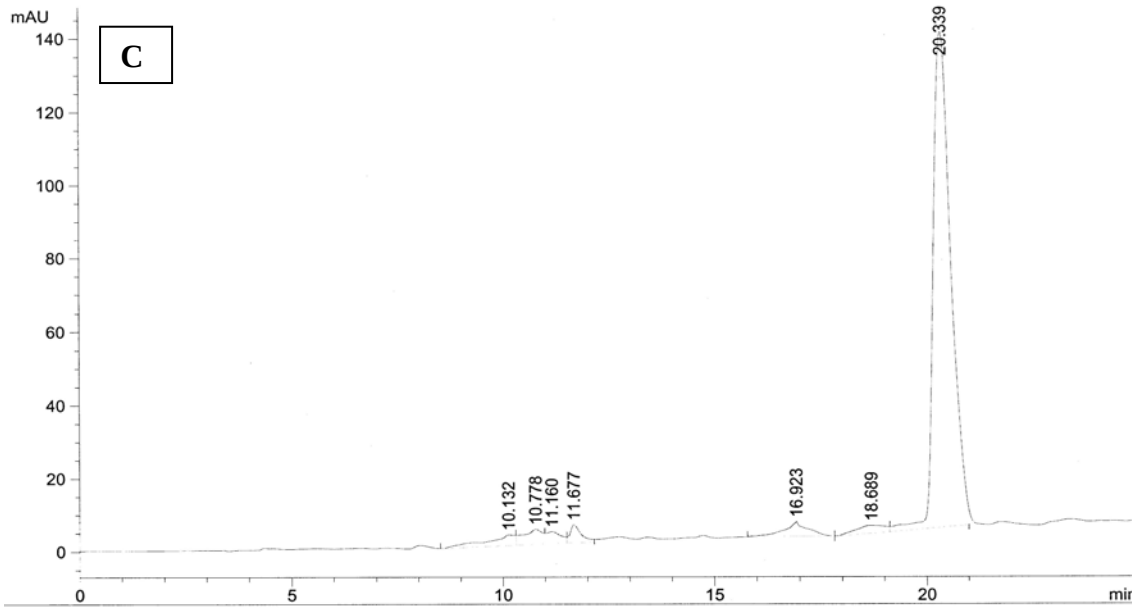
Appendix A

HPLC chromatograms for GTP (A), GMP (B) and c-di-GMP (C) standards showed that GTP was eluted at 18.9 min, GMP at 12.1 min and c-di-GMP at 20.3 min. Peak heights for GTP, GMP and c-di-GMP standards were used to obtain the calibration curves for these nucleotides.






C
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140
120
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10.132
10.778
11.160
11.677
16.923
18.689
20.339
0
5
10
15
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min

Appendix B

Raw data for LDH assay

The cytotoxicity effect of the bacteria on mammalian cells were evaluated by the amount of lactate dehydrogenase released in the supernatant measured with Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN). Maximum release was achieved by lysis of cells with 1% Triton-X. LDH activity in supernatant of uninfected cells was taken as spontaneous release. Percentage cytotoxicity was calculated with the formula:

$$\% \text{ cytotoxicity} = \frac{(\text{Test LDH release} - \text{spontaneous release})}{(\text{Maximal release} - \text{spontaneous release})}$$

	Reading 1	**% cytotoxicity**	Reading 2	**% cytotoxicity**	Reading 3	**% cytotoxicity**	Reading 4	**% cytotoxicity**	Reading 5	**% cytotoxicity**	Reading 6	**% cytotoxicity**	**Average**	SD
KHW *B. pseudomallei*	0.908	**33.709**	0.935	**35.737**	1.092	**43.392**	1.097	**44.632**	0.979	**34.442**	0.944	**31.930**	**37.31**	5.352
KHW*cdpA::Tet* mutant	0.390	**4.510**	0.398	**7.051**	0.396	**7.180**	0.391	**7.298**	0.340	**4.086**	0.390	**6.293**	**6.07**	1.423
*KHW*cdpA::Tet/pUCP28T-*cdpA*	0.955	**36.359**	0.987	**38.515**	0.991	**38.137**	1.012	**40.137**	1.002	**35.534**	0.984	**33.781**	**37.08**	2.294
KHW/pUCP28T-cdpA	0.932	**35.062**	0.937	**35.844**	1.032	**40.271**	1.021	**40.613**	0.991	**35.012**	0.104	**40.945**	**37.96**	2.928
KHWBPSS0805::Km mutant	1.044	**41.375**	1.033	**40.972**	0.998	**38.502**	1.136	**46.695**	0.909	**31.116**	1.023	**35.585**	**39.04**	5.344
KHWBPSS0805:: Km/pUCP28T-BPSS0805	0.985	**38.050**	0.946	**36.325**	1.035	**40.427**	1.008	**39.926**	1.082	**39.335**	1.043	**36.511**	**38.43**	1.749
KHW/pUCP28T-BPSS0805	0.901	**33.315**	1.032	**40.919**	0.988	**37.981**	0.982	**38.551**	1.028	**36.770**	0.957	**32.531**	**36.68**	3.216
High control	2.084		2.138		2.180		2.144		2.359		2.415			
Low control	0.310		0.266		0.258		0.253		0.254		0.254			